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TITLE: Down-Regulation of Olfactory Receptors in Response to Traumatic Brain Injury Promotes Risk for Alzheimer's Disease

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14. ABSTRACT:

Background: Traumatic Brain Injury (TBI) is a risk factor for subsequent development of Alzheimer's disease (AD). Abnormal tau processing is a common pathological feature of TBI and AD, and tau neuropathology plays a key role in both TBI complications and AD dementia. We recently found aberrant down-regulation of specific olfactory receptors (ORs) as biological indices for TBI. Moreover, our feasibility evidence suggests that that down-regulation of OR TBI biomarkers following TBI may contribute to TBI-related tau neuropathology.
Purpose: Our proposed studies were designed to investigate whether down-regulation of select OR TBI biomarkers in the brain may contribute to the elevation of tau neuropathological phenotypes, thereby promoting the development of AD dementia among Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) veterans with exposure to TBI.
Scope: Our proposed studies were designed to be executed with two Aims. Our proposed Specific Aim 1 studies were designed to develop neuronal culture systems with overexpression of specific OR TBI biomarkers and then, using these novel *in vitro* experimental models, to screen for selective pharmacological OR activators and to investigate the role of OR biomarkers in tau processing. Our proposed Specific Aim 2 studies were designed to use a recently developed blast-induced mild TBI (bTBI) rat model to investigate the association between exposure to TBI, OR expression and abnormal tau processing, *in vivo*.
Results: We also observed, in PBMCs of bTBI rats, down regulation of several ORs (Olr1671, Olr322 and Olr735 that, respectively, are rodent orthologs of human OR2J3, OE4D9 and OR4X1) that we previously found down-regulated in PBMCs of humans with TBI. Our evidence demonstrated a cause-effect relationship between TBI exposure and OR down-regulation. We found that activation of OR TBI biomarkers, such as OR4M1, may reduce tau neuropathological phenotypes, in part, by inhibiting cellular JNK signaling, thereby interfering with the phosphorylation of tau at select residues that are associated with tauopathies. Outcomes from our study demonstrated that abnormal tau processing in subjects with a history of TBI may be traced back to abnormal down-regulation of certain OR TBI biomarkers including OR4M1 and OR11H1, in the brain. Our studies also identified a novel high-affinity OR4M1 agonist and demonstrated that in primary neuronal cultures, activation of OR4M1 by this agonist significantly reduced cellular tau phosphorylation.
Significance: Collectively, our findings support our hypothesis that inactivation of certain ubiquitous ORs in response to conditions associated with TBI might be at the basis for potential abnormal tau phosphorylation, possibly through mechanisms associated with the JNK pathway. This evidence suggests a potential fine-tuning character of OR-mediated JNK regulation in response

15. SUBJECT TERMS
Nothing listed

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1. INTRODUCTION

Traumatic Brain Injury (TBI) is an acquired injury caused by sudden trauma to the head which disrupts normal brain functioning and leads to either transient or chronic impairments in physical, cognitive, emotional, and/or behavioral functions. In the civilian population, TBI is typically associated with direct, close impact mechanical trauma to the brain due to falls, motor vehicle accidents and sports. In contrast, TBI among military personnel, particularly among veterans returning from the Persian Gulf region as part of service in Operation Enduring Freedom (OEF) or Operation Iraqi Freedom (OIF), is primarily due to exposure to blast pressure waves stemming from blast-producing weaponry, such as improvised explosive devices (IEDs). There is growing evidence of persistent long-term impacts of TBI on cognition. In particular, there is strong epidemiological evidence (Van Den et al., 2007) and growing pathophysiological (Johnson et al., 2010) and experimental (Sivanandam and Thakur, 2012) evidence linking prior exposure to traumatic brain injury (TBI) to increased risk for development of Alzheimer's disease (AD). However, the details of the biological interrelationship between TBI exposure and long-term complications such as AD, how to identify individuals with TBI who are at a higher risk for these long-term complications, and how to alter the prognosis of those individuals are poorly understood. We recently found among a civilian TBI study cohort that exposure to TBI is associated with long-term aberrant down-regulation of specific olfactory receptors (ORs) (e.g., OR11H1, OR4M1 and OR52N5) in peripheral blood mononuclear cells (PBMCs) (Zhao et al., 2013). In addition, we found down-regulation of these ORs is significantly correlated with the severity of brain injury and TBI-specific symptoms, and a two-biomarker panel, comprised of OR11H1 and OR4M1, provides the best criterion for segregating the TBI and control cases, with 90% accuracy, 83.3% sensitivity, and 100% specificity (Zhao et al., 2013; Appendix 1). More importantly, we also found lower contents of these ORs in select brain regions among cases who have suffered from TBI, suggesting that down-regulation of these ORs in the brain following TBI might contribute to TBI-related neuropathological alterations leading to long-term neurological complications, such as AD. Both AD and TBI exhibit tau neuropathology, which plays a key role in TBI complication and AD dementia. Based on our *in vitro* feasibility evidence suggesting activation of our OR TBI biomarkers modulates tau neuropathology-related phenotypes we hypothesized that presence and activation of ectopically expressed OR TBI biomarkers in the brain may protect against abnormal tau processing and that down-regulation of OR TBI biomarkers in response to TBI may lead to tau neuropathology, thereby promoting the development of AD dementia among OIF/OEF veterans with exposure to TBI. Based on this, our overall study is designed to explore whether down-regulation of ORs post-TBI may contribute to long-term TBI complications such as AD, and clarify the underlying mechanisms for this. The overall study is separated into three parts: *in vitro* studies using primary neuron culture to identify high affinity, selective ligands for OR TBI biomarkers OR4M1, OR11H1 and OR52N5; *in vitro* study using these high-affinity OR ligands to investigate the impacts of OR downstream signaling on tau neuropathogenic mechanisms in primary neuron cultures; and *in vivo* assessment of OR and tau signaling in a blast exposure rodent model of mild (m) TBI.

2. KEYWORDS

Olfactory receptors, mild traumatic brain injury, Alzheimer's disease, tau neuropathology, *in silico* drug screening, tau phosphorylation, JNK signaling

3. ACCOMPLISHMENTS

- ***What were the major goals of the project?***

Major goals of the projects, specified as Tasks 1 and 2 in the Statement of Work, are as follow:

TASK 1. To Continue to Characterize OR Signal Transduction Pathways Associated with Attenuation of TBI Neuropathology (Months 1-18).

A. Screening a larger odorant library to identify individual compounds that are able to selectively activate OR11H1, OR4M1 or OR52N5 with high affinity. (Months 1-6).
   - 100% completion

B. Identifying cellular signals that are associated with OR-mediated modulation of tau neuropathology (months 6-18).
   - 100 % completion

TASK 2. To Continue to Explore the Association between Exposure to TBI, OR Expression and Abnormal Tau Processing in Experimental Rodent Models (Months 13-24).

A. Assessing if blast TBI in a rodent model may promote tau neuropathology over time and whether induction and progression of tau neuropathology might be associated with down-regulation of OR TBI biomarkers (Months 13-24)
   - 100 % completion

- ***What was accomplished under these goals?***

  Accomplishments under the purview of Task 1 and 2 are summarized below:

  Accomplishments under Task 1A:
  Identification of candidate high-affinity, selective OR4M1 ligands via virtual receptor modeling and in silico ligand screening.

  1. We initially identified several low-affinity OR4M1 agonists that are able to activate OR4M1 at 10 μM concentrations (Zhao et al., 2013; Appendix 1). To screen for OR4M1 agonist with higher affinity, we constructed a virtual 3D structure for OR4M1, from which we identified a ligand binding pocket as an initial target for high throughput, 3-D *in silico* screening against virtual compound libraries for the identification of candidate small molecular OR4M1 ligands. As we have discussed in our Second Year Final Report, we conducted an *in silico* screening against approximately 5 million lead-like compounds (Teague et al., 1999) from the ZINC library of commercially available compounds and identified 57 candidate OR4M1 ligands. The identified compounds were grouped into 32 clusters based on chemical similarities. Based on the availability of these compounds, we chose 25 commercially available compounds for experimental validation. In parallel studies, we also generated a NSE-OR4M1 transgenic mouse line with overexpression of OR4M1 in brain neurons (Zhao et al, 2015 Provisionally accepted; Appendix 2), which provided us with a standardized and consistently reproducible source of primary cortico-hippocampal neuron cultures with overexpression of OR4M1 for testing bioactivity of candidate OR4M1 ligands. As we have discussed in our Second Year Final Report and in Zhao et al., 2015 (Provisionally Accepted; Appendix 2) we tested the candidate OR4M1 ligands using NSE-OR4M1 primary cortico-hippocampal neuron cultures and identified multiple OR4M1 agonists that are capable of interacting with and activating OR4M1, as evident by observations of ligand-mediated induction of cellular cAMP, an early and key component of cellular signal transduction by ORs. Among the identified novel OR4M1 agonist, ZINC10915775 was particularly efficient in the activation of OR4M1 as indicated by increases in cellular cAMP contents in response to ligand treatment, inducing OR4M1 signaling at nM concentrations with maximal OR4M1 activation at 100 nM concentration (Zhao et al., 2015, Provisionally Accepted; Appendix 2). Consistent with our working hypothesis that activation of OR TBI biomarkers in the brain reduces tau neuropathology, we observed that treatment of NSE-OR4M1 primary cortico-hippocampal neuron cultures with the OR4M1 agonist Z221560030

significantly reduced cellular Tau phosphorylation (Zho et al., 2015, Provisionally Accepted; Appendix 2). While our observation specifically highlighted beneficial effects of OR4M1 activation by pharmacological means, our observation also support the possibility that pharmacological activation of other OR TBI biomarkers, such as OR11H1 or OR52N5, might also similarly attenuate tau neuropathological phenotypes.

Based on information relating to chemical structures and OR4M1-activiting bioactivity of the small molecular ligands we generated from our initial screening process, we further refined our *in silico* screening model system to optimize the efficacy of the model system for identifying candidate OR4M1 ligands with improved affinity and specificity. As we have discussed in our Second Year Final Report, we used this refined model in an *in silico* screening against virtual compounds from the ZINC database, as well as additional compounds from our in-house FSL library of drug-like compounds and the NCI Open library of compounds. We identified 64 candidate small molecular OR4M1 ligands that might be more potent and selective than ZINC10915775 for activating OR4M1 for future pharmacological development.

2. Using a strategy similar to our screening program for OR4M1 agonists, we constructed a virtual 3D structural model for OR11H1 and identified a receptor binding pocket, which we used as a target for 3D *in silico* screening against virtual compounds from the ZINC library. As we have discussed in our Second Year Final Report, we identified 83 candidate small molecular OR11H1 ligands that showed good occupancy of the predicted binding pocket. We found the 83 compounds can be grouped into 7 clusters of structurally similar compounds; the remaining 64 compounds did not fall into clusters. These compounds provide key targets for future pharmacological development of select OR11H1 agonists for modulating Tau neuropathology phenotypes in veteran and civilian subjects with TBI and/or AD.

Accomplishments under Task 1B:

Identifying cellular signals underlying modulation of tau neuropathology by OR TBI biomarkers.

1. To assess the potential role of OR4M1 on tau phosphorylation in brain neuronal cells, we designed and constructed a lentiviral OR4M1 expression plasmid and packaged the construct into OR4M1 lentiviral particles capable of overexpressing OR4M1 in mammalian cells. We then transduced primary cortico-hippocampal neuron cultures with purified OR4M1 lentiviral particles to induce neuronal overexpression of OR4M1. Using OR4M1 lentivirus transduced primary neuron cultures, we screened known odorants for their potential to interact with and activate OR4M1 by monitoring for odorant-mediated induction of cAMP as an index of OR4M1 activation. We identified several low-affinity OR4M1 agonists that are able to activate OR4M1, but only in the presence of high, μM concentrations of the agonists (Zho et al., 2013; Appendix 1). We continued and investigated effects of OR4M1 activation on tau neuropathological phenotypes using one of the identified low-affinity OR4M1 agonists, acetophenone. We found that treatment of OR4M1 lentivirus transduced primary cortico-hippocampal neuron cultures with 10 μM of acetophenone significantly reduced tau phosphorylation, in part, by inhibiting cellular JNK signaling that plays a key role in tau hyperphosphorylation and that is known to be activated by various forms of stress (Zho et al., 2013; Appendix 1).

   The cause-effect relationship between neuronal activation of OR4M1 and protection against tau neuropathological phenotypes were further examined using NSE-OR4M1 primary cortico-hippocampal neuron cultures and the higher-affinity OR4M1 agonist, ZINC10915775. Consistent with our evidence using the low-affinity OR4M1 agonist acetophenone, we found that treatment with 100 nM Z221560030 significantly decreased tau phosphorylation on Ser202/Thr205 (AT8 epitope) and also on Thr212/Ser214 (AT100 epitope), but not on Ser396/404 (PHf1-epitope) (Zhao et al, 2015, Provisionally

Accepted; Appendix 2). Moreover, we observed that these modulatory effects of Z221560030 on neuronal tau phosphorylation are mediated, in part, by the inhibition of cellular JNK signaling cascade in response to OR4M1 activation (Zhao et al, 2015, Provisionally Accepted; Appendix 2).

Accomplishments under Task 2A:
Explore the Association between TBI exposure, OR expression and abnormal tau processing in vivo.

1. Based on our initial observation from a civilian mTBI study cohort implicating OR11H1, OR4M1 and OR52N5 mRNA as biomarkers for TBI, we continued and investigated regulations of the three OR TBI biomarkers using banked PBMCs from a cohort of five OEF/OIF veteran and a control cohort of seven age- and gender-matched veterans with TBI (non-TBI veterans) (Zho et al., 2013; Appendix 1). Similar to our observation from the civilian study cohort, we found all three OR TBI biomarkers are significantly down-regulated in PBMC specimens of veteran TBI cases compared to that of non-TBI control cases (Zho et al., 2013; Appendix 1). Continuing to explore the potential relevance of OR11H1, OR4M1, and OR52N5 to TBI clinical symptoms, we found that the contents of the three OR biomarkers in PBMCs were significantly and inversely correlated with TBI severity: lower OR mRNA contents in PBMCs are associated with exposure to more severe head injury (Fig. 4A–C). We also found that OR TBI biomarker contents in PBMCs were significantly correlated with select aspects of self-reported clinical TBI symptoms. In particular, PBMC contents of OR11H1 or OR4M1 were significantly associated in an inverse fashion with TBI-specific symptoms, a summation of 25 cognitive symptoms that are sensitive and specific to TBI (Zhao et al., 2013; Appendix 1). OR11H1 or OR4M1 content within PBMCs were not associated with self-assessments of mood (Fig. 4D). Expression level of the third OR biomarker, OR52N5, was not correlated with TBI-specific symptoms or self-assessment of mood (Zhao et al., 2013; Appendix 1).

2. We continued and explored the potential physiological relevance of OR11H1, OR4M1, and OR52N5 in the brain using post-mortem brain specimens. We found the three TBI biomarker ORs are expressed in multiple regions of the brain, including temporal gyrus, entorhinal cortex, occipital cortex, and the hippocampal formation (Zhao et al., 2013; Appendix 1). Our observation that the three OR biomarkers were ectopically expressed in multiple brain regions outside of the olfactory bulb suggested potential function(s) of these ORs in the brain unrelated to the detection and processing of olfactory information.

3. We continued and explored the potential inter-relationship between TBI exposure and down-regulation of ORs in a blast-induced mTBI (bTBI) rat model that was initially developed by Chavko et al. 2006 (Chavko et al., 2006). We monitored the expression of ORs in bTBI rats longitudinally, for up to 12 month after blast exposure. Similar to what we previously found in subjects with a history of TBI (Zhao et al., 2013; Appendix 1), we found significantly lower contents of Olr1612, the rodent ortholog of human OR4M1, in PBMCs of bTBI rats compared to age- vender-matched control rats without blast treatment. We also observed, in PBMCs of bTBI rats, down regulation of several ORs (Olr1671, Olr322 and Olr735 that, respectively, are rodent orthologs of human OR2J3, OE4D9 and OR4X1) that we previously found down-regulated in PBMCs of humans with TBI (Zhao et al., 2013; Appendix 1). Collectively, our evidence demonstrated a cause-effect relationship between TBI and OR down-regulation.

- ***What opportunities for training and professional development has the project provided?***
  Nothing to report
- ***How were the results disseminated to communities of interest?***

Results were reported in two scientific research manuscripts for publication in peer-reviewed journals. One manuscript, Zhao et al., 2013 (Appendix 1) was published in *Journal of Alzheimer's Disease*. The second manuscript, Zhao et al., 2015 (Appendix 2) has been provisionally accepted to the *Journal of Cellular Biochemistry*.

We have also reported our studies in scientific meetings:

Pasinetti, GM. Attenuation of neuropathological features shared by TBI and AD through olfactory receptor activation. *Arrowhead's 3rd Annual TBI Conference*, Washington D.C., USA, March, 2013

Pasimetti, GM. Role of olfactory receptors in Traumatic Brain Injury-associated tauopathy. *Society of Biological Psychiatry 67th Annual Convention*, Philadelphia, PA, USA, May, 2012

Pasinetti GM, Gordon W, Ho L, Zhao W. Role of olfactory receptor in Traumatic Brain Injury-Associated Tauopathy. *Ninth World Congress on Brain Injury,* Edinburgh, Scotland, March, 2012.

Pasinetti GM, Gordon W, Dams-O'Connor K, Zhao W, Ho L. Traumatic Brain Injury (TBI) induces down-regulation of olfactory receptors that are ectopically expressed in the brain: implications in TBI-mediated tauopathy. *Experimental Biology Meeting*, San Diego, CA, USA, April, 2012.

- ***What do you plan to do during the next reporting period to accomplish the goals?***
  Nothing to Report

4. **IMPACT** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- **What was the impact on the development of the principal discipline(s) of the project?**

The overall goal of the project was to investigate whether down-regulation of select OR TBI biomarkers in the brain may contribute to the elevation of tau neuropathological phenotypes, thereby promoting the development of AD dementia among Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) veterans with exposure to TBI. Outcomes from our study demonstrated that abnormal tau processing in subjects with a history of TBI may be traced back to abnormal down-regulation of certain OR TBI biomarkers, including OR4M1 and OR11H1, in the brain (Zhao *et al.*, 2013). We also demonstrated that activation of OR TBI biomarkers, such as OR4M1 may reduce tau neuropathological phenotypes, in part, by inhibiting cellular JNK signaling, thereby interfering with the phosphorylation of tau at select residues that are associated with tauopathies. Interestingly the physiological relevance of OR4M1 is consistent with the recent study demonstrating that controlled cortical impact TBI activates JNK and increases tau phosphorylation in a 3xTg mouse model of AD (Tran et al., 2011). Our studies also identified a novel high-affinity OR4M1 agonist and demonstrated that in primary neuronal cultures, activation of OR4M1 by this agonist significantly reduced cellular tau phosphorylation. Collectively, our findings support our hypothesis that inactivation of certain ubiquitous ORs in response to conditions associated with TBI might be at the basis for potential abnormal tau phosphorylation possibly through mechanisms associated with the JNK pathway. This evidence suggests a potential fine-tuning character of OR-mediated JNK regulation in response to conditions associated with TBI.

Our observation from the experimental bTBI model demonstrated, for the first time, a direct cause-and effect relationship between long-term down regulation of select ORs and a neuropathological feature in TBI. This validates our hypothesis that down regulation of select ORs in PBMCs and in the brains of our human TBI study cohort may be caused by prior TBI exposure (Zhao *et al.*, 2013; Appendix 1). Our 3-D *in silico* screening

model provides an innovative means to identify novel compounds that can modulate tau phosphorylation through activation of ORs. Future studies will focus on structure-activity relationships (SAR) around the active compound and the optimization of an *in silico* screening system based on this information to identify compounds with higher potency and specificity as potential preventative and possibly therapeutic agents for TBI associated pathologic phenotypes at early stages.

- **What was the impact on other disciplines?**

Results from studies demonstrating activation of OR TBI biomarkers reduces tau neuropathology support further development of select OR agonists as novel therapeutics for modulating tau neuropathology in TBI, AD, as well as in other tauopathies such as progressive supranuclear palsy, chronic traumatic encephalopathy, frontotemporal dementia and parkinsonism linked to chromosome17, among others.

- **What was the impact on technology transfer?**

Our 3-D *in silico* screening model provides an innovative means to identify novel compounds that can modulate tau phosphorylation through activation of ORs. Future studies will focus on structure-activity relationships (SAR) around the active compound and the optimization of an *in silico* screening system based on this information to identify compounds with higher potency and specificity as potential preventative and possibly therapeutic agents for TBI associated pathologic phenotypes at early stages.

- **What was the impact on society beyond science and technology?**

Increasing epidemiological evidence demonstrated prior exposure to TBI is associated with increased risk for AD. Outcomes from our studies provide novel mechanistic supports linking TBI exposure and AD, thereby strengthening the association between TBI exposure and risk for AD. This information will help raises awareness among both civilians and military personnel of the long-term complications from, which will provide the impetus to mobilize resources and or political will to develop novel strategies that would minimize TBI exposure and/or that would improve outcomes following TBI exposure.

5. **CHANGES / PROBLEMS**

- **Changes in approach and reasons for change**
  Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  We requested for and received a no cost extension of the project to from 24 to 30 months for us to complete our proposed studies.

- **Changes that had a significant impact on expenditures**
  Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  Nothing to report

- **Significant changes in use or care of human subjects**
  Nothing to report

- **Significant changes in use or care of vertebrate animals.**
  Nothing to report

- **Significant changes in use of biohazards and/or select agents**
  Nothing to report

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

Journal publications.

Zhao W, Ho L, Varghese M, Yemul S, Dams-O'Connor K, Gordon W, Knable L, Freire D, Haroutunian V, Pasinetti GM. Decreased level of olfactory receptors in blood cells following traumatic brain injury and potential association with tauopathy. J Alzheimers Dis., 2013;34(2):417-29.

Zhao W, Ho L, Wang J, Bi W, Yemul S, Ward L, Freire D, Mazzola P, Brathwaite J1, Mezei M, Roberto Sanchez R, Elder GA, Pasinetti GM. In Silico Modeling of Olfactory Receptor 4M1 and Biochemical Characterization of Novel Ligands in Tauopathy Attenuation. Journal of Cellular Biochemistry, 2015 (Provisionally Accepted).

Books or other non-periodical, one-time publications.
Nothing to report

- **Other publications, conference papers, and presentations.**

Pasinetti, GM. Attenuation of neuropathological features shared by TBI and AD through olfactory receptor activation. *Arrowhead's 3rd Annual TBI Conference*, Washington D.C., USA, March, 2013

Pasimetti, GM. Role of olfactory receptors in Traumatic Brain Injury-associated tauopathy. *Society of Biological Psychiatry 67th Annual Convention*, Philadelphia, PA, USA, May, 2012

Pasinetti GM, Gordon W, Ho L, Zhao W. Role of olfactory receptor in Traumatic Brain Injury-Associated Tauopathy. *Ninth World Congress on Brain Injury*, Edinburgh, Scotland, March, 2012.

Pasinetti GM, Gordon W, Dams-O'Connor K, Zhao W, Ho L. Traumatic Brain Injury (TBI) induces down-regulation of olfactory receptors that are ectopically expressed in the brain: implications in TBI-mediated tauopathy. *Experimental Biology Meeting*, San Diego, CA, USA, April, 2012.

- **Website(s) or other Internet site(s)**
  Nothing to report

- **Technologies or techniques**
  Our OR4M1 lentivirus and our NSE-OR4M1 transgenic mouse line are available for investigators by other scientists, upon request.

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	*Giulio Maria Pasinetti*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36*
Contribution to Project:	*Oversaw the design and execution of the overall study, interpretation of the data gathered and preparation of reports and manuscripts.*
Funding Support:	

Name:	*Lap Ho*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36*
Contribution to Project:	*Conducted gene expression of PBMCs from the civilian and the veteran TBI study cohort leading to the identification of OR TBI biomarkers, conducted the studies exploring the regulation of OR biomarkers in the bTBI rat model, and helped with the design of the overall studies, interpretation of the data and preparation of the manuscripts*
Funding Support:	

Name:	*Wei Zhao*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*30*
Contribution to Project:	*Conducted the investigations on distributions of OR TBI biomarkers in the brain, prepared the OR4M1 lentiviral virus, conducted the in silico screening studies toward identification of candidate ligands for OR4M1 and OR11H1. Also conducted the studies exploring OR4M1-activating bioactivities of candidate OR ligands, effects of OR4M1 activation on tau phosphorylation in primary neuronal cultures, and molecular mechanisms by which activation of OR4M1 reduces tau neuropathology. Helped with the execution of the studies exploring the regulation of OR biomarkers in the bTBI rat model, the design of the overall studies, interpretation of the data and preparation of the manuscripts*
Funding Support:	

Name:	*Merina Varghese*
Project Role:	*Co-investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Assisted with the execution of the studies exploring effects of OR4M1 activation on tau phosphorylation in primary neuronal cultures, and molecular mechanisms by which activation of OR4M1 reduces tau neuropathology.*
Funding Support:	

Name:	*Hanna Reding*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Assisted with the execution of the studies exploring the regulation of OR biomarkers in the bTBI rat model.*
Funding Support:	

Name:	*Jun Wang*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Assisted with the execution of the studies exploring the regulation of OR biomarkers in the bTBI rat model, design of the overall studies, interpretation of the data and preparation of the manuscripts.*
Funding Support:	

Name:	*Bing Gong*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Assisted with the execution of the in silico screening studies toward identification of candidate ligands for OR4M1 and OR11H1*
Funding Support:	

Name:	*Lauren Dubner*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Assisted in the conduct of the in silico screening studies toward identification of candidate ligands for OR4M1 and OR11H1*
Funding Support:	

Name:	*Anindita Bose*
Project Role:	*Post-doctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month	*1*

worked:	
Contribution to Project:	*Helped conduct the in silico screening studies toward identification of candidate ligands for OR4M1 and OR11H1*
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  No.

- **What other organizations were involved as partners?**
  None

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS**
  Nothing to report

- **QUAD CHARTS:**
  Not applicable

9. **APPENDICES:**

Appendix 1: Zhao W, Ho L, Varghese M, Yemul S, Dams-O'Connor K, Gordon W, Knable L, Freire D, Haroutunian V, Pasinetti GM. Decreased level of olfactory receptors in blood cells following traumatic brain injury and potential association with tauopathy. J Alzheimers Dis., 2013;34(2):417-29.

Appendix 2: Zhao W, Ho L, Wang J, Bi W, Yemul S, Ward L, Freire D, Mazzola P, Brathwaite J1, Mezei M, Roberto Sanchez R, Elder GA, Pasinetti GM. In Silico Modeling of Olfactory Receptor 4M1 and Biochemical Characterization of Novel Ligands in Tauopathy Attenuation. Journal of Cellular Biochemistry, 2015 (Provisionally Accepted).

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Decreased Level of Olfactory Receptors in Blood Cells Following Traumatic Brain Injury and Potential Association with Tauopathy

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Abstract. Traumatic brain injury (TBI) is a leading cause of death and disability among children and young adults in the United States. In this study, we explored whether changes in the gene expression profile of peripheral blood mononuclear cells (PBMC) may provide a clinically assessable "window" into the brain, reflecting molecular alterations following TBI that might contribute to the onset and progression of TBI clinical complications. We identified three olfactory receptor (OR) TBI biomarkers that are aberrantly down-regulated in PBMC specimens from TBI subjects. Down-regulation of these OR biomarkers in PBMC was correlated with the severity of brain injury and TBI-specific symptoms. A two- biomarker panel comprised of OR11H1 and OR4M1 provided the best criterion for segregating the TBI and control cases with 90% accuracy, 83.3% sensitivity, and 100% specificity. We found that the OR biomarkers are ectopically expressed in multiple brain regions, including the entorhinal-hippocampus system known to play an important role in memory formation and consolidation. Activation of OR4M1 led to attenuation of abnormal tau phosphorylation, possibly through JNK signaling pathway. Our results suggested that addition of the two-OR biomarker model to current diagnostic criteria may lead to improved TBI detection for clinical trials, and decreased expression of OR TBI biomarkers might be associated with TBI-induced tauopathy. Future studies exploring the physiological relevance of OR TBI biomarkers in the normal brain and in the brain following TBI will provide a better understanding of the biological mechanisms underlying TBI and insights into novel therapeutic targets for TBI.

Keywords: Biomarker, olfactory receptor, peripheral blood mononuclear cell, tauopathy, traumatic brain injury

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INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability among children and young adults in the United States [1]. TBI is an acquired injury caused by a sudden trauma to the head that disrupts

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normal brain functioning, which leads to either transient or chronic impairments in physical, cognitive, emotional, and/or behavioral functions. In the civilian population, TBI is typically associated with direct, closed impact mechanical trauma to the brain due to falls, motor vehicle accidents, sports, etc. [2]. In contrast, TBI among military personnel, particularly among veterans returning from the Persian Gulf region, is primarily due to exposure to blast pressure waves stemming from blast-producing weaponry, leading to prototypical cognitive deficits including impairments in attention, memory, processing speed, and executive functioning [2–4]. Both civilians and veterans who suffer from TBI exhibit symptoms that range in severity from mild to very severe, with a minimal to profound impact on daily functioning. The reasons why TBI induces different clinical symptoms among affected individuals are not yet known.

New evidence has highlighted defects in neural circuit and synapses, and the plastic processes controlling these functions, in TBI [5–11]. While genes relevant to these processes are expressed in the brain, some of these genes are also expressed in circulating blood cells, such as peripheral blood mononuclear cells (PBMCs) [12–15]. Consistent with this, recent studies have illustrated that PBMC-associated biomarkers may provide insights into the pathogenesis of neurological disorders such as Alzheimer's disease and can be used to monitor disease diagnosis and progression [16, 17]. Thus, PBMC may also provide an ideal, clinically assessable "window" into the brain, reflecting molecular alterations following TBI which might contribute to the onset and progression of clinical TBI phenotypes.

Evidence suggests that appropriate interventions can reduce functional impairment after TBI [18–20]. In order to demonstrate the efficacy of clinical interventions, research must identify the biological, clinical, and neurological indexes that are sensitive to the detection of functional impairments after TBI. Therefore, in this study we explored the feasibility of identifying clinically assessable TBI biomarkers and the potential function of identified TBI biomarkers in TBI neuropathology.

MATERIALS AND METHODS

Civilian TBI and control subjects

Eleven individuals with TBI (five male and six female), with TBI severity ranging from mild to severe, and nine control participants (four male and five female) were recruited from the Brain Injury Research Center at the Mount Sinai School of Medicine (MSSM). Medical documentation of TBI was reviewed for each participant. The age- and education-matched controls were recruited using advertisements placed in local media and flyers, and through word of mouth. Following a capacity screening to assess the individual's ability to comprehend the purpose and procedures of the study and provide informed consent, the informed consent process was performed in accordance with MSSM IRB policies and procedures.

All participants were screened using the Brain Injury Screening Questionnaire (BISQ) [21, 22] to assess the kinds of situations in which a brain injury might have occurred, and the number and severity of hits to the head sustained throughout the lifespan. Using information gained from the BISQ and from individual interviews conducted by the authors, injury severity was classified using a 7-point scale ranging from 1 (no loss of consciousness, no confusion (i.e., no TBI)) to 7 (loss of consciousness greater than 4 weeks in duration) [21, 22]. For those who have been injured, questionnaires in the BISQ solicit participants' self-reported measures of functional difficulties and symptoms associated with brain injury and events, conditions other than brain injury that might lead to symptoms similar to those seen in brain injury, as well as the occurrence of persistent, highly disruptive symptoms (functional changes) that do not "go away", which TBI participants may continue to experience following the blow to the head.

Veteran TBI and control subjects

For this biomarker validation study, we obtained banked PBMCs from a cohort of five OEF/OIF veterans and a control cohort of seven age- and gender-matched veterans without TBI (non-TBI veterans). TBI diagnosis is based on confirmation according to Defense and Veterans Brain Injury Center (DVBIC) criteria of sustained injury to the head plus subsequent alteration of consciousness, and Repeatable Battery for Neuropsychological Testing (RBANS) scores of one standard deviation below the norms established for age and education of individuals in question. Non-TBI control classification is based on DVBIC confirmation of no sustained injury to the head and RBANS scores less than one standard deviation below the norms established. Most of the veteran TBI cases exhibit comorbid post-traumatic stress disorder (PTSD). Thus, our non-TBI control cases are also matched for PTSD

diagnosis. In this study, diagnosis of PSTD is based on a score of 50 or more in the PTSD Checklist – Civilian Version.

Postmortem brain specimens

Human postmortem brain samples from 4 neurologically normal cases (characterized by a Clinical Dementia Rating (CDR) of 0) were obtained from the Alzheimer's Disease Brain Bank of the Mount Sinai School of Medicine. A multistep approach based on cognitive and functional status during the last 6 months of life was applied to the assignment of CDR [23] as previously reported [24, 25].

PBMC and RNA isolation

Blood specimens were collected into BD Vacutainer CPT Cell Preparation Tubes. PBMC were isolated following manufacturer's instructions (Becton, Dickinson and Company) and were stored at −80°C until use. Total RNA was isolated from approximately 10–50 mg of PBMC using RNA STAT-60 (Tel-Test) according to the manufacturer's instructions. The purity and concentration of RNA samples were determined from OD260/280 readings using a dual beam UV spectrophotometer and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies).

Microarray study and analysis

Total RNA was directly labeled using the FlashTag™ HSR Biotin RNA Labeling Kit according to the manufacturer's instructions (Genisphere). Verification of biotin labeling was obtained by an enzyme-linked oligoabsorbant assay (ELOSA) using Immobilizer™ Amino – 8 well strips (Nunc/Thermo Fisher Scientific) according to instructions supplied by Genisphere. Labeled RNA (1.0 μg) was hybridized for 16 h at 48°C to Affymetrix HuGene 1.0 ST arrays containing probe sets for 28,869 genes. Arrays were washed and stained on a Fluidics Station 450 (Affymetrix) according to the manufacturer's recommended procedures. The arrays were stained with phycoerythrein-conjugated streptavidin (Life Technologies) and the fluorescence intensities were determined using a GCS 3000 7G high-resolution confocal laser scanner and AGCC software (Affymetrix). The scanned images were analyzed with the RNA QC tool (Affymetrix) using RMA global background correction, quantile normalization and median polish summarization to generate quantified data (as recommended by Genisphere). Quality control metrics for arrays included normalized signal values >1000 for five spike-in control oligo probe sets (Genisphere).

RNA probe sets exhibiting significant differential expression (SDE) were identified using the following steps in GeneMaths XT (Applied Maths): 1) Probe sets with array detection p-values ≤ 0.05 for all samples in at least one experimental group were selected for further analysis; 2) Performed Discriminant Analysis (DA) and determined the largest percentage of remaining probe sets that permitted correct group assignment of samples in unsupervised hierarchical clustering by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) based on cosine correlation of row mean centered log2 signal values; this was the top 50 percentile; and 3) in the DA top 50 percentile, selected probe sets with absolute signal log2 fold changes ≥ 1.0 and independent t-test p-values ≤ 0.05 adjusted for multiple testing error by the Benjamini-Hochberg FDR correction method. Unsupervised hierarchical clustering of probe sets and heat map generation were performed in GeneMaths XT following row mean centering of log2 transformed MAS5.0 signal values; probe set clustering was performed by the UPGMA method using Cosine correlation as the similarity metric. For comparative purposes, clustered heat maps included probe sets for spike-in controls (Genisphere), or endogenous small RNAs exhibiting: 1) Array detection p-values ≤ 0.05, and 2) either a) a log2 signal value standard deviation ≤ 0.025 for all samples or b) in the DA top 50 percentile with a FC >1.3 in the opposite direction of the selected SDE profile.

Confirmatory quantitative PCR

First strand cDNA was synthesized from 1 μg of total RNA using Superscript III Super-mix for qRT-PCR (Invitrogen). Quantitative RT-PCR was performed using Maxima SYBR Green master mix (Fermentas) in ABI Prism 7900HT in four replicates with primers amplifying the seven olfactory receptor candidates: OR4Q3 (Forward: CACCTGCTCCAATC TCCTATG, Reverse: TCCCCTAACATCTTTGGCA C); OR51L1 (Forward: TTCCCACACCTTTGCTA CTG, Reverse: AATACTGTTGGTCCTGGCATC); OR4D10 (Forward: CCATCTCTGTCACCTTCAC TG, Reverse: ATGGCTGACTTCATCTCATGG); OR4M1 (Forward: TCTGTTAATGTCCTATGC-CTTCC, Reverse: AATGTGGGAATAGCAGGTGG);

OR52N5 (Forward: ATGCTACCACACTCACCAAC, Reverse: CATCAGCAATACACCCCTCAG); OR2J3 (Forward: CCTCTCATCCTCATTCTCACTTC, Reverse: CAAACACTTTCTGAAGCCCAG); OR11H1 (Forward: AACTGGTCATACTGTGCTGG, Reverse: GGGCAGAAACACAATCCAATG). Human TATA-binding protein (TBP, Forward: TGCACAGGAGCC AAGAGTGAA, Reverse: CTGGAACGGTGAAG-GTGACA) expression level was used as an internal control. Data were normalized using the $2^{-\Delta\Delta Ct}$ method [26]. Levels of olfactory receptor mRNAs were expressed relative to those in control groups and plotted in GraphPad Prism.

Lentiviral plasmid construction and lentivirus packaging

We obtained pCMV6-XL5-OR4M1 cDNA clone from Origne and subcloned the OR4M1 ORF into lentivial plasmid pLVX-IRES-ZsGreen (Clontech). The inserted OR4M1 sequence was verified by sequencing. For lentivirus packaging, we transfected Lenti-X 293T cells with either pLVX-OR4M1-IRES-ZsGreen or pLVX-IRES-ZsGreen using Lentiviral Packaging System (Clontech). Medium was collected 48 h after transfection and a ten-fold concentration step was performed using Lenti-X Concentrator (Clontech).

Primary neuron culture, lentiviral transduction, and cAMP assay

Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from C57BL6 mouse (Jackson Laboratory) as previously described [27]. Cells were seeded onto poly-D-lysine-coated 12-well plates at 5×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, and 1% penicillin-streptomycin (Life Technologies). On Day 5 of culture, primary cortico-hippocampal neuron cultures were transduced with lentiviral particles overexpressing OR4M1 or control lentiviral particles by spin infection (800 g × 90 min at 30°C). Transduction efficiency was monitored by ZsGreen expression. 72 h after lentiviral transduction, cells were pretreated with 3-isobutyl-l-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, for 10 min followed by ligand treatment (10 μM) for 10 min. cAMP assay was performed using a colorimetric cAMP ELISA assay kit (Cell Biolabs).

Luminex multiplex assay and western blot analysis

Primary cortico-hippocampal neurons were infected with OR4M1 lentiviral particles and stimulated with acetophenone (10 μM) for 1 h. Multiplex luminex assay was performed using the Milliplex xMAP 8-plex multipathway signaling-phosphoprotein kit (Millipore) as previously described [28]. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific). 30 μg of total protein were loaded onto 12% SDS-PAGE and subjected to western blot analysis with antibody recognizing phospho-JNK (T183/Y185) (Cell Signaling), phospho-ERK (T185/Y187) (Cell Signaling), and PHF-1 (phospho-tau (S396/S404), a generous gift from Peter Davies at Albert Einstein College of Medicine). Blots were quantified in ImageJ (NIH), normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies) and then plotted in GraphPad Prism.

RESULTS

Identification of select olfactory receptors as clinically accessible candidate TBI biomarkers

We analyzed the gene expression profile in PBMC derived from subjects with TBI and age-, gender-, and education-matched healthy controls. Our data showed that female subjects had a more robust profile than males probably due to a rather smaller number of male subjects in the study cohort. Taking this into consideration, further analysis and confirmatory studies were all performed in a subset of the study cohort which only contains female subjects. Demographic information for the female TBI and control participants is summarized in Table 1. Using microarray gene expression profile analysis, we identified a panel of 102 candidate biomarker genes (36 annotated and 66 non-annotated genes) that are differentially regulated (*t*-test $p < 0.05$) following Benjamini-Hochberg false discovery rate correction [29] by >1.5-fold in PBMC specimens of TBI cases compared to control cases (Fig. 1A). More importantly, we were able to correctly separate all cases into TBI and non-TBI groupings using this panel of 102 genes in an unsupervised hierarchical clustering analysis (Fig. 1A). Interestingly, among the panel of 102 candidate TBI biomarkers that we identified from microarray data, 7 were olfactory receptors (Fig. 1B). Our microarray studies revealed that each of the 7 ORs were down-regulated in PBMC

Table 1
Demographic information for the civilian study cohort. TBI severity was classified using a 7-point scale ranging from 1 (no loss of consciousness, no confusion (i.e., no TBI)) to 7 (loss of consciousness greater than 4 weeks in duration) [23]. Time post injury is the time frame between the occurrence of brain injury and volunteer's participation in this biomarker study

	Control	TBI
Number of cases	5	6
Gender	100% female	100% female
TBI severity		
Mean ± SD	1 ± 0	4 ± 1.67
Range	1	2–6
Age (mean y ± SD) at study participation	34.80 ± 14.48	35.33 ± 13.32
Post injury interval (mean y ± SD)	—	5.42 ± 5.30
Education (mean y ± SD)	16.80 ± 1.10	14.50 ± 2.35
Ethnicity composition		
White-Caucasian	80%	66.7%
Black-African American	—	33.3%
Hispanic	20%	—

of TBI cases compared to those of control cases in this study cohort (Figs. 1B, 2A).

Confirmatory qPCR studies

Using independent qPCR assays, we confirmed that three of the ORs (OR11H1, OR4M1, and OR52N5) were significantly down-regulated in PBMC of TBI cases (Fig. 2B). For the remaining four ORs (OR4D10, OR2J3, OR4Q3, OR51L1), our qPCR evidence showed lower levels of these ORs in PBMC of TBI compared to control cases, but these differences did not reach statistical significance (Fig. 2B). This particular result is conceivably due to a high degree of gene expression variability for these 4 ORs among healthy control cases and the relatively small sample sizes that we used in our exploratory study.

We continued to explore the sensitivity and specificity of an individual or combined role of OR11H1, OR4M1, and OR52N5 in distinguishing TBI cases from normal healthy controls in our study cohort (Fig. 2C). Using unsupervised clustering analyses, we found that a two- biomarker panel comprised of OR11-H1 and OR4M1 provides the best criterion for segregating the TBI and control cases with 90% accuracy, 83.3% sensitivity, and 100% specificity (Fig. 2C, D).

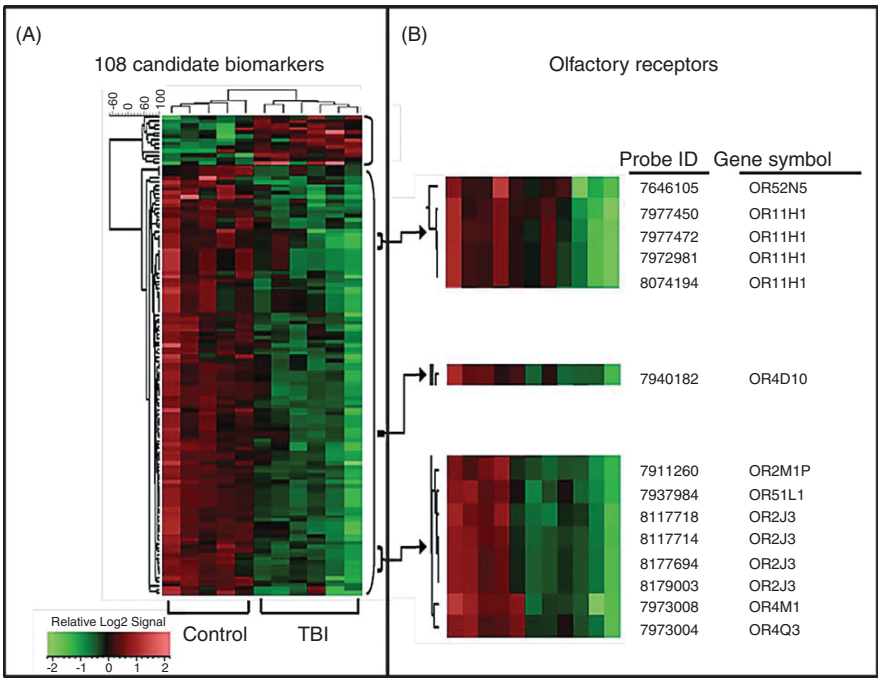


Fig. 1. Development of blood biological indices (biomarkers) capable of correctly segregating TBI and control cases. Gene expression profile analysis of PBMC specimens from TBI and 5 age-, gender-, and education-matched control cases using a microarray platform (Affymetrix) led to the identification of a panel of 102 candidate biomarker genes. A) The 108 differentially-regulated genes identified were subjected to unsupervised hierarchical clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are presented as a heat map (left panel) demonstrating that the content of the 108 biomarker panel is able to correctly segregate TBI from control cases. B) 7 ORs and one OR pseudogene that are down-regulated in PBMC of TBI cases.

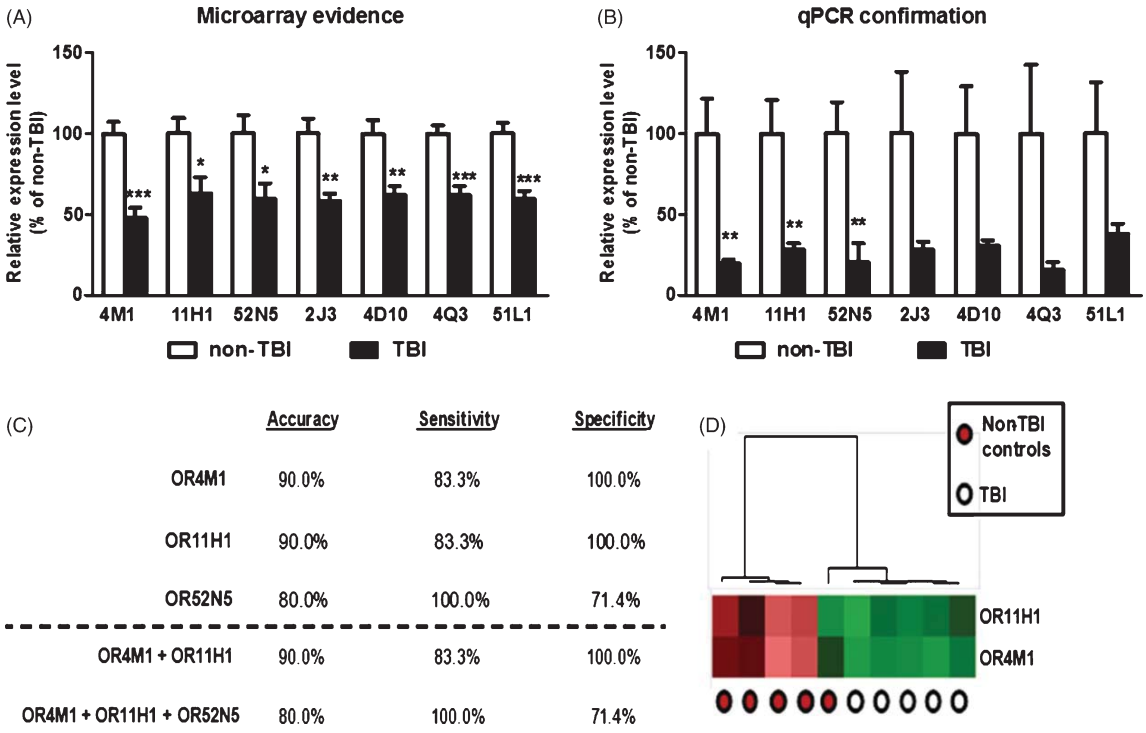


Fig. 2. Down-regulation of OR genes in PBMC of TBI cases provide a sensitive and specific criterion for distinguishing TBI from control cases. mRNA contents for each of the 7 candidate OR biomarker genes in TBI and control cases were analyzed by microarray (A) or independent qPCR (B). Bar graphs represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by student t-test, TBI versus control. The efficacy of using biomarker contents from PBMC as a criterion to correctly segregate TBI and control cases was tested by unsupervised clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. C) The accuracy, sensitivity, and specificity of OR11H1, OR4M1, OR52N5, or panels of ORs to distinguish TBI from control cases. D) A heat map graphically depicting the efficacy of using the two biomarker panel to distinguish TBI cases and control cases by unsupervised clustering analysis.

TBI biomarker validation studies: Testing the validity of OR11H1, OR4M1, and OR52N5 to distinguish TBI from non-TBI control cases from a study cohort of OEF/OIF veterans

The pathophysiological mechanisms underlying mechanical and blast-related TBI may differ in some ways, but they share important pathophysiological features. Similarities between the pathophysiologies of mechanical and blast-related TBI [2] suggested that information gathered from TBI cases in the civilian population may also be relevant to combat-related TBI. Based on this, we tested the ability of OR11H1, OR4M1, and OR52N5 to distinguish TBI cases from non-TBI controls from a study cohort of OEF/OIF veterans. For this biomarker validation study, we obtained banked PBMCs from a cohort of five OEF/OIF veterans and a control cohort of seven age- and gender-matched veterans without TBI (non-TBI veterans) (Table 2). Similar to our above-mentioned observations regarding OR biomarkers from the civilian study cohort, we found that OR4M1 (Fig. 3A), OR11H1 (Fig. 3B), and OR52N5 (Fig. 3C) are also significantly down-regulated in PBMC specimens of veteran TBI cases compared to that of non-TBI control cases. The two biomarker panel of OR11H1 and OR4M1 we identified from the civilian TBI study cohort is also capable of distinguishing veteran TBI from control veteran cases with 83% accuracy, 80% sensitivity, and 86% specificity (Fig. 3D & E).

OR biomarker contents in PBMCs are correlated with TBI severity & long-term clinical neuropsychological complications

Continuing to explore the potential relevance of OR11H1, OR4M1, and OR52N5 to TBI clinical symptoms, we found that the contents of the three OR biomarkers in PBMC was significantly and inversely correlated with TBI severity: lower OR mRNA

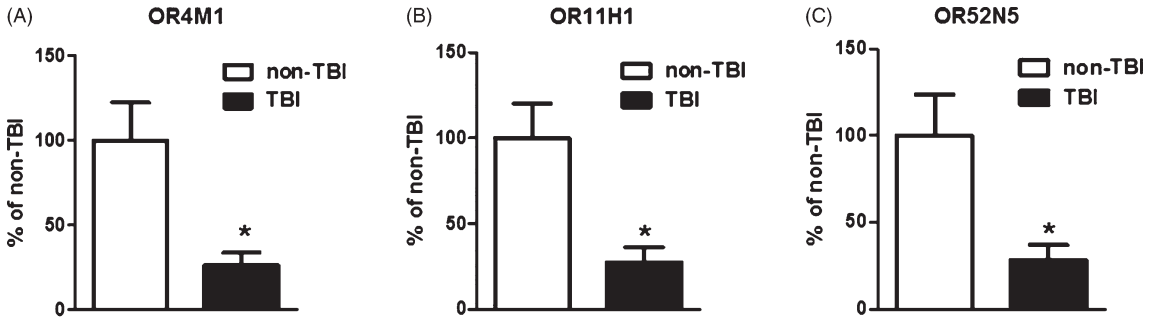
Table 2
Demographic information for the veteran study cohort. TBI diagnosis is based on confirmation according to Defense and Veterans Brain Injury Center (DVBIC) criteria of sustained injury to the head plus subsequent alteration of consciousness, and Repeatable Battery for Neuropsychological Testing (RBANS) scores of one standard deviation below the norms established for age and education of individuals in question. Non-TBI control classification is based on DVBIC confirmation of no sustained injury to the head and RBANS scores less than one standard deviation below the norms established. Most of the veteran TBI cases exhibit co-morbid post-traumatic stress disorder (PTSD). Thus, our non-TBI control cases are also matched for PTSD diagnosis. In this study, diagnosis of PSTD is based on a score of 50 or more in the PTSD Checklist – Civilian Version

	Control	TBI
Number of cases	7	5
Gender	57% male	80% male
Age (mean y ± SD)	30.1 ± 9.4	31.0 ± 7.1
Interval since last deployment (mean y ± SD)	2.8 ± 2.3	4.0 ± 3.8
Education (mean y ± SD)	13.3 ± 2.8	13.8 ± 1.5
Percentage of cases with co-morbid PTSD	85.7%	100%
Ethnicity composition		
Black-African American	14.3%	40%
Hispanic	85.7%	60%

contents in PBMCs are associated with exposure to more severe head injury (Fig. 4A–C). We also found that OR TBI biomarker contents in PBMC were significantly correlated with select aspects of self-reported clinical TBI symptoms. In particular, PBMC contents of OR11H1 or OR4M1 were significantly associated in an inverse fashion with TBI-specific symptoms, a summation of 25 cognitive symptoms that are sensitive and specific to TBI [22]. OR11H1 or OR4M1 content within PBMC were not associated with self-assessments of mood (Fig. 4D). Expression level of the third OR biomarker, OR52N5, was not correlated with TBI-specific symptoms or self-assessment of mood (Fig. 4D).

Ectopic expression of OR TBI biomarkers in multiple brain regions outside of the olfactory bulb

The brain represents a key target tissue for understanding TBI clinical complications and for the development of clinical interventions. In order to explore the potential physiological relevance of OR11H1, OR4M1, and OR52N5 in the brain, we assessed the RNA contents of OR biomarkers in the brain. We found that the three TBI biomarker ORs were expressed in multiple regions of the brain from post-mortem brain specimens, including temporal gyrus (BM22), entorhinal cortex (BM36), occipital cortex (BM17), and the hippocampal formation (Fig. 5A & B). In agreement with our RT-PCR data, we also



(D)

	Accuracy	Sensitivity	Specificity
OR4M1	83.0%	80.0%	86.0%
OR11H1	83.0%	80.0%	86.0%
OR52N5	67.0%	67.0%	67.0%
OR4M1+OR11H1	83.0%	80.0%	86.0%
OR4M1+OR11H1+OR52N5	75.0%	80.0%	78.3%

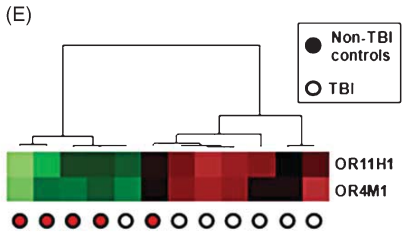
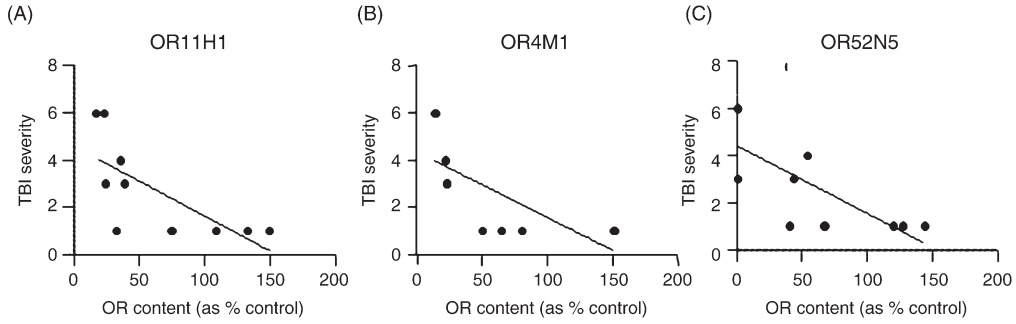


Fig. 3. Validation of OR TBI biomarkers in a veteran study cohort. The mRNA contents for (A) OR4M1, (B) OR11H1, and (C) OR52N5 in TBI and control cases from a veteran study cohort were analyzed by qPCR. Bar graphs represent mean + SEM. *$p < 0.05$ by student *t*-test, TBI versus control. D) The accuracy, sensitivity, and specificity of OR11H1, OR4M1, OR52N5, or panels of ORs to distinguish veteran TBI from control veteran cases. E) A heat map graphically depicting the efficacy of using the two biomarker panel to distinguish veteran TBI cases and control veteran cases by unsupervised clustering analysis.



(D)

	TBI severity		TBI-specific symptoms		Mood	
	Correlation coefficient	p-value	correlation coefficient	p-value	correlation coefficient	p-value
Olfactory Receptor 11H-1	-0.709	0.022	-0.711	0.021	-0.449	NS
Olfactory Receptor 4M-1	-0.72	0.019	-0.733	0.016	-0.26	NS
Olfactory Receptor 52N-5	-0.757	0.011	-0.518	NS	-0.251	NS

Fig. 4. Olfactory receptor TBI biomarker contents in PBMCs are inversely correlated with TBI severity and long-term neuropsychological complications. Correlation analysis was used to test the potential association between PBMC OR11H1, OR4M1, and OR52N5 mRNA content and the severity of TBI injury or self-reported measures of TBI complications. OR11H1 (A), OR4M1 (B), and OR52N5 (C) content in PBMCs are inversely correlated with TBI severity. D) Correlation coefficients and *p*-values of associations between individual OR biomarker content in PBMCs and TBI severity, TBI-specific symptoms (a summation of 25 cognitive symptoms that are sensitive and specific to TBI) and self-assessment of mood.

found the expression of OR TBI biomarkers in the hippocampal formation region from the human brain genome-wide microarray database [30] (Fig. 5C–E). Our observation that the three OR biomarkers were ectopically expressed in multiple brain regions outside of the olfactory bulb suggested potential function(s) of these ORs in the brain unrelated to the detection and processing of olfactory information.

Activation of OR TBI biomarker modulates tau neuropathology-related phenotypes in vitro

Olfactory receptors are members of the class A rhodopsin-like family of G protein-coupled receptors (GPCRs). Once the odorant has bound to the odor receptor, the receptor undergoes structural changes and it binds and activates the olfactory-type G protein on the inside of the olfactory receptor neuron. The G protein (Golf and/or Gs) in turn activates adenylate cyclase, which converts ATP into cyclic AMP (cAMP). So we first examined the expression level of Golf and β-arrestin in the PBMC of TBI and control subject and no change was detected (Supplementary Figure 1; available online: http://www.j-alz.com/issues/34/vol34-2.html#supplementarydata04).

Considering that tau neuropathology is a main pathological feature following TBI, we continued to explore whether OR4M1 activation might influence tau processing mechanisms. We transduced primary cortical-hippocampal neuron culture with OR4M1 lentiviral particles and screened several odorants for their ability to activate OR4M1 using cAMP ELISA assay. In Fig. 6A, we presented some representative odorants that were capable or not capable of activating OR4M1. Positive ligands were used to stimulate OR4M1-overexpressing primary cortico-hippocampal neurons and the potential impact of activation of OR4M1 on signaling pathways and aberrant tau phosphorylation were assessed. Using luminex multiplex technology, we found that upon acetophenone stimulation, phosphorylation of c-Jun N-terminal kinase

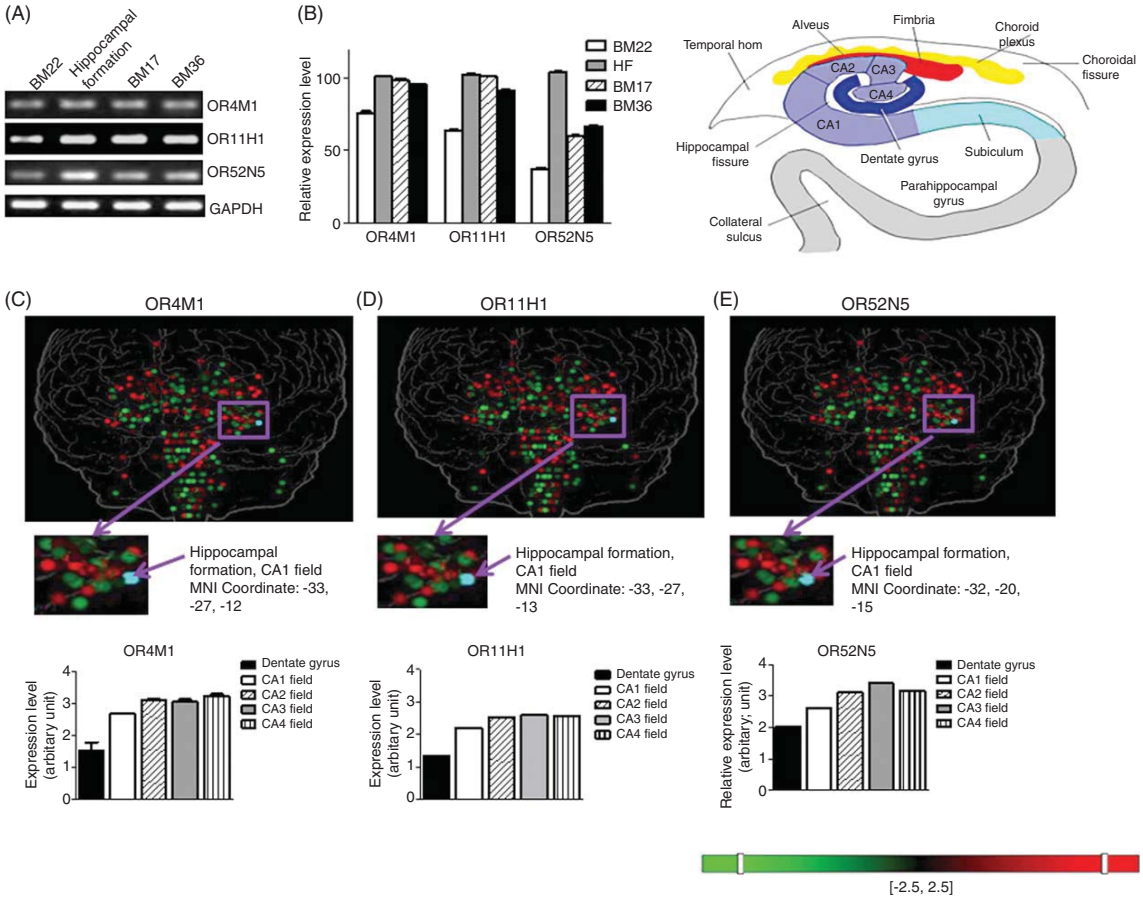


Fig. 5. Olfactory receptor TBI biomarkers expression in the brain. OR11H1, OR4M1, and OR52N5 mRNA expression by (A) RT-PCR and (B) relative expression level in postmortem superior temporal gyrus (BM22), hippocampal formation (HF), occipital cortex (BM17), and entorhinal cortex (BM36) specimens from neurologically normal cases. Expression pattern of (C) OR4M1, (D) OR11H1, and (E) OR52N5 in the brain by genome-wide microarray from the Allen Human Brain Atlas [30].

(JNK) at Thr183/Tyr185 was significantly reduced in neurons overexpressing OR4M1 (Fig. 6B), indicating the activation of OR4M1 might influence JNK signaling pathway. The phosphorylation of ERK1/2 (Fig. 6B), among others (STAT3, MEK, p70S6, IKBa, and CREB, see Supplemental Figure 2), did not change upon acetophenone stimulation, suggesting that this inhibition of JNK signaling is rather specific. The stress-activated kinase JNK belongs to the mitogen-activated protein kinase family and takes part in signaling cascades initiated by various forms of stress. Its targets include the microtubule-associated protein tau [31]. Hyperphosphorylation of tau at Ser396/Ser404 is a known feature associated with tau neuropathology in neurodegenerative disorders such as Alzheimer's disease. Encouragingly, we observed that overexpression and activation of OR4M1 in primary neurons significantly reduced cellular content of abnormally phosphorylated tau at Ser396/Ser404 (PHF1 epitope) (Fig. 6C), suggesting that activation of OR4M1 might result in protection against abnormal tau processing.

Collectively, our data demonstrated that select olfactory receptors (e.g., OR11H1, OR4M1, and OR52N5) were down-regulated in the PBMC of TBI cases and could serve as TBI biomarkers. Activation of OR4M1 resulted in protection against tau neuropathological features possibly through the JNK signaling pathway (Fig. 7).

DISCUSSION

Consistent with accumulation evidence suggesting that PBMC-associated biomarkers may provide

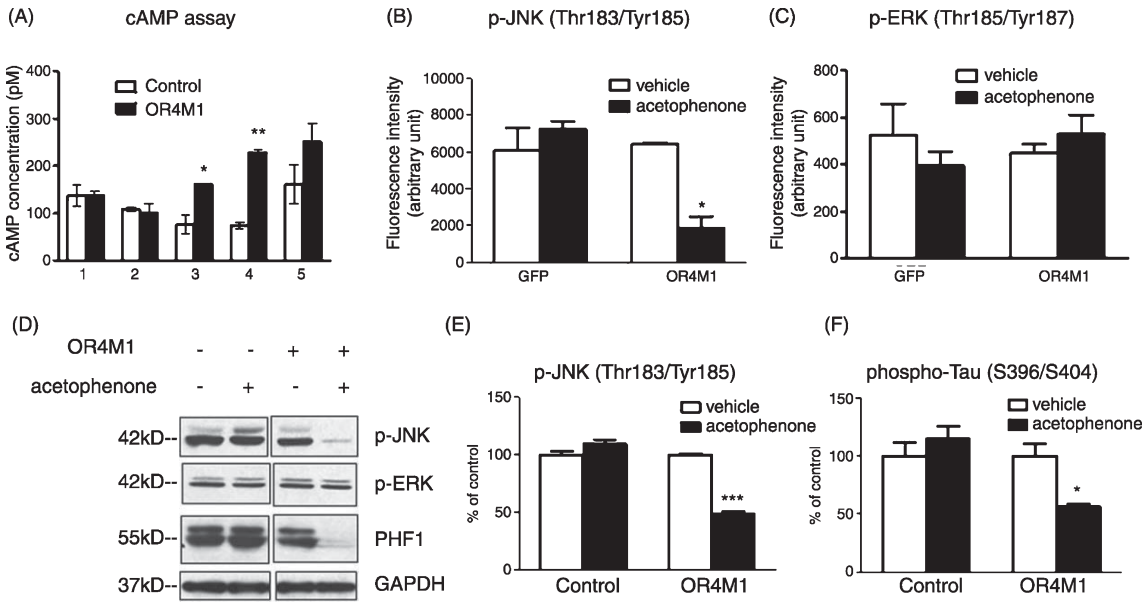


Fig. 6. Activation of OR4M1 resulted in reduced tau phosphorylation via JNK signaling pathway. Primary cortico-hippocampal neuron culture was transduced with lentiviral particles overexpressin OR4M1 or control lentiviral particles by spin infection (250 g × 90 min at 30°C). A) Transduced cells were treated by (1) hedione, (2) (+) citronellal, (3) acetophenone, (4) pyrazine, and (5) 2-isobutyl-3-methopyrazine (all at 10 μM, Sigma-Aldrich) for 10 min and cAMP assay was performed. Transduced neurons were also treated with acetophenone for 1 h, and multiplex luminex assay was performed using the Milliplex xMAP 8-plex multipathway signaling-phosphoprotein kit (Millipore) according to manufacturer's protocol: (B) Change of JNK phosphorylation on Thr183/Tyr185 and (C) ERK1/2 phosphorylation on Thr185/Tyr187. JNK and tau phosphorylation were also measured by (D) western blot analysis using antibodies recognizing phospho-JNK, phospho-ERK, or phospho-tau (PHF-1 epitope) and quantified (E, F) using GAPDH as a loading control. *p<0.05, **p < 0.01, ***p < 0.001 by two-tailed student t-test.

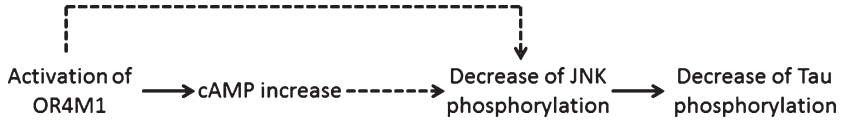


Fig. 7. Scheme of working hypothesis.

insights into the pathogenesis of neurological disorders, results from our studies revealed that expression of select ORs in PBMC may serve as clinically assessable surrogate biological indices of TBI. ORs are G protein-coupled receptors known to be expressed in nasal epithelium olfactory neurons, where they are responsible for the detection of odorants [32]. However, ectopic expression of select ORs, defined as a biological event or process that occurs in an abnormal location or position in the brain or other tissues, has also been described [33]. Our high throughput microarray evidence, followed by data from independent qPCR confirmatory studies, led to the identification of three OR TBI biomarkers (OR11H1, OR4M1, and OR52N5) that are ectopically expressed in PBMC and are aberrantly down-regulated in PBMC specimens from subjects with a history of TBI. Among the TBI cases in our study cohort, there was a significant time lag between the occurrence of brain injury and the volunteers' participation in this biomarker study; the average post-injury interval among the TBI cases was 5.4 ± 5.3 years (Table 1). Thus, down-regulation of the three ORs in PBMC among TBI cases was reflections of long-term physiological consequences of TBI.

We found that down-regulation of the three OR biomarkers in PBMC were directly correlated with the severity of brain injury in our TBI participants. Among the three OR biomarkers, we found that a two-biomarker panel, comprised of OR11H1 and OR4M1, provides the best criterion for segregating the TBI and control cases with 90% accuracy, 83.3% sensitivity, and 100% specificity. Interestingly, we found that PBMC contents of OR11H1 and OR4M1 were inversely correlated with cognitive-related symptoms.

While additional studies will be required to clarify the mechanisms underlying the inter-relationships between changes in OR biomarker contents in PBMC, the initial severity of brain injury, and long-term clinical consequences of TBI, outcomes from our studies suggest that additional applications of this two-OR biomarker panel to current diagnostic criteria may lead to improved TBI detection and more sensitive outcome measures for clinical trials.

Initial and persistent cognitive deficits are the most common complaints after TBI [34, 35]. The entorhinal-hippocampus system is known to play an important role in the formation and consolidation of memories, particularly spatial memories [36–38]. Among the brain regions surveyed, we found the highest content of OR TBI biomarkers in the hippocampal formation, with relatively lower levels of OR TBI expression in the entorhinal cortex. Based on this and on our observation that the down-regulation of OR11H1 and OR4M1 in the PBMC are inversely correlated with self-reported indexes of cognitive functions, it is likely that ectopic expression of OR biomarkers in the entorhinal-hippocampus circuitry might be relevant to long-term deficits in cognitive functions following TBI.

It is well known that TBI may be a risk factor for dementia [39], but recent evidence suggests that Alzheimer's disease-related neuropathological mechanisms may contribute to cognitive dysfunction in TBI. The two characteristic neuropathologies of Alzheimer's disease are the abnormal accumulation and deposition of amyloid-β peptides and tau proteins in the brain. Evidence from humans [40–42] and experimental animal models [43] has also revealed abnormal accumulations of amyloid-β peptides and tau proteins in the brain and in cerebrospinal fluid following TBI. Interestingly, elevation of plasma tau levels has been associated with increasingly severe outcomes of TBI [44]. Multiple signaling pathways have been demonstrated to be involved in TBI pathology, including the AKT signaling pathway [45], glycogen synthase kinase-3 signaling pathway [46], STAT3 signaling pathway [47], ERK signaling pathway [48], and JNK signaling pathway [49]. We found that activation of OR4M1 could lead to attenuation of abnormal tau phosphorylation on S396/404 via JNK signaling pathway, suggesting a possible link between OR4M1 and TBI-related tauopathy. Further studies will be needed to investigate abnormal tau phosphorylation level in the serum/cerebrospinal fluid of TBI subjects, especially the phosphorylation of Thr181, which has been implicated as a fluid-based marker in dementia [50]. Studies will also be needed to dissect in detail the signaling pathways that are associated with the attenuation of tau neuropathology through OR activation.

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Keywords:	traumatic brain injury, olfactory receptor, G protein-coupled receptors, in silico screening, tau phosphorylation, rodent model of mild traumatic brain injury

In Silico Modeling of Olfactory Receptor 4M1 and Biochemical Characterization of Novel Ligands in Tauopathy Attenuation

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Running title: OR4M1 Ligands Attenuate Tauopathy

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Keywords

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- olfactory receptor
- G protein-coupled receptors
- in silico screening
- tau phosphorylation
- rodent model of mild traumatic brain injury

Abstract

Traumatic brain injury (TBI) is a risk factor for several neurodegenerative disorders. We previously reported that abnormal tau processing, a canonical feature in Alzheimer's disease and other tauopathies, may be traced back to abnormal down-regulation of certain ubiquitous olfactory receptors (ORs) in the brain, eg., the OR4M1 subtype, in subjects with a history of TBI. The objective of this study was to investigate the role of OR in TBI mediated tau pathology and the feasibility of using OR ligands as a novel therapeutic intervention. We built a 3-D OR model and used *in silico* screening of potential novel ligands from commercial drug libraries able to functionally activate OR signaling and eventually attenuate the formation of abnormal tau. Excitingly we found that *in vitro* activation of OR4M1 with the commercially available ZINC library compound 10915775 led to a significant attenuation of abnormal tau phosphorylation in embryonic cortico-hippocampal neuronal cultures derived from NSE-OR4M1 transgenic mice, possibly through modulation of the JNK signaling pathway. The attenuation of abnormal tau phosphorylation was rather selective since ZINC10915775 significantly decreased tau phosphorylation on tau Ser202/T205 (AT8 epitope) and tau Thr212/Ser214 (AT100 epitope), but not on tau Ser396/404 (PHF-1 epitope). Moreover, no response of ZINC10915775 was found in control hippocampal neuronal cultures derived from wild type littermates. Our *in silico* model provides novel means to pharmacologically modulate select ubiquitously expressed ORs in the brain through high affinity ligand activation to prevent and eventually to treat TBI-induced down regulation of ORs and subsequent cascade of tau pathology.

Traumatic brain injury (TBI) is an acquired injury caused by a sudden trauma to the head which disrupts normal brain functioning, leading to either transient or chronic impairments in physical, cognitive, emotional, and/or behavioral functions. In the civilian population, TBI is typically associated with direct, closed impact mechanical trauma to the brain due to falls, motor vehicle accidents, sports, *etc.* (Elder & Cristian 2009b). In contrast, TBI among military personnel, particularly among Veterans returning from the Persian Gulf region as part of service in Operation Iraqi Freedom (OIF) or Operation Enduring Freedom (OEF), is primarily due to exposure to blast pressure waves stemming from blast-producing weaponry, such as improvised explosive devices (IEDs) (Elder & Cristian 2009a).

We previously reported decreased expression of select olfactory receptors in subjects with TBI history (Zhao et al. 2013a) and found an association between abnormal tau formation and decreased expression of certain ubiquitously expresses ORs in the brain and in circulating mononuclear cells. ORs are members of the class A rhodopsin-like family of G protein-coupled receptors (GPCRs). While ORs have been largely investigated for their role in olfaction, little is known about their role in the brain. For example, it is well known that specific odorants, when bound to select ORs in the olfactory mucosa, may transduce the sensation through OR-type G proteins consistent with the general concept that GPCRs, like olfactory ORs are membrane-bound proteins that are responsible for cell-environment communications (Horn et al. 1998). Moreover, recent evidence suggests conditions associated with inflammation, obesity, hypertension as well as mood disorders such as anxiety and depression are also associated with altered GPCRs (Wilson & Bergsma 2000), making them among the most important targets for pharmacological intervention (Rattner, Sun, & Nathans 1999;Sautel & Milligan 2000;Schoneberg, Schulz, & Gudermann 2002). Unfortunately, GPCRs, like other membrane-embedded proteins, have characteristics that make their 3-D structure difficult to determine experimentally. While the structures of several GPCRs have been determined recently, no experimental structures of ubiquitously expressed ORs are currently available.

The current study is designed to identify ligands for the ubiquitously expressed OR4M1 via in silico screening using a homologous model of OR4M1 and to discover potential novel receptor ligands able to be further develop as future new treatment of abnormal tau processing in TBI and other neurodegenerative disorders.

Materials and Methods

Homology Modeling and Binding Site Identification

A homology model of OR4M1 was built using MODELLER version 9.12 (Eswar et al. 2003) using bovine rhodopsin as a template. Because the sequence similarity between OR4M1 and bovine rhodopsin is below 25% identity, the alignment of the two sequences was built using profiles of homologous olfactory receptors and other GPCRs. The profiles were matched to each other using the SALIGN routine of program MODELLER (Marti-Renom, Madhusudhan, & Sali 2004). The alignment was manually edited using predictions of transmembrane helices for OR4M1. The quality of the alignment was evaluated by mapping predicted olfactory receptor binding site residues (Man, Gilad, & Lancet 2004a) onto the model. A divergent loop (Ala175-Pro175) was remodeled using the loop modeling routine of MODELLER (Fiser, Do, & Sali 2000). A total of 100,000 conformations for the loop were generated and the best scoring one, based on the DOPE potential (Shen & Sali 2006), was selected as the final model. The ligand-binding pocket in the final model was defined by the known OR binding site residues and by the SiteHound binding site identification program (Ghersi & Sanchez 2009b).

Docking, Scoring and Selection

The pocket identified in the OR4M1 model was used as a target for virtual screening of small molecular compounds. A set of ~ five million lead-like compounds (Teague et al. 1999) derived from the ZINC library of commercially available compounds (Irwin & Shoichet 2005b) was docked into the binding site using program DOCK version 6.5 (Moustakas et al. 2006). Lead-like compounds were selected to facilitate optimization of validated hits. Compounds for experimental testing were selected based on docking score and visual inspection of binding site complementarity and hydrogen bonding. Clustering of compounds based on chemical fingerprint similarity was carried out using single-linkage clustering and using a Tanimoto coefficient of 0.7 as the cutoff. Chemical fingerprint similarity was computed using the JChem software (ChemAxon).

Experimental Animals

Rat neuron-specific enolase (NSE) promoter plasmid containing human OR4M1 was constructed by inserting ~1kb cDNA fragment with the entire coding region of hOR4M1 (NM_001005500.1, OriGene Technologies, Inc. Rockville, MD) in the NotI site of the plasmid vector. A cassette of ~6 kb Sal1 fragment containing NSE promoter and hPGC-1α was gel purified and microinjected into one-cell mouse egg (C57BL6 x SJL) as described previously (Kelley et al. 1999;Qin et al. 2006). TgOR4M1 founders were identified by PCR-based genotyping. All animals were maintained on a 12:12-h light/dark cycle with lights on at 07:00 h in a temperature-controlled (20 ± 2°C) vivarium, and all procedures were approved by the MSSM IACUC.

Mild TBI (mTBI) rodent models were generated by blast exposure, as previously described (Chavko, Prusaczyk, & McCarron 2006). Briefly, adult male Long Evans hooded rats (250–350g; 10–12 weeks of age) were used as subjects. Rats were exposed to overpressure injury using the Walter Reed Army Institute of Research (WRAIR) shock tube, which simulates the effects of air blast exposure under experimental conditions (Elder et al. 2012). Blood samples were withdrawn through the saphenous vein 1, 3, 6, and 12 months following the blast. At the end of the study, the cortex, hippocampus and cerebellum were collected and stored at -80°C until further analysis.

Primary neuron preparation, treatment and cAMP assay

Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from TgOR4M1 mice. Neurons were seeded onto poly-D-lysine-coated 12-well plates at 5×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5mM L-glutamine, and 1% penicillin-streptomycin (all from Life Technologies). On Day 5 of the culture, primary cortico-hippocampal neuron cultures were pretreated with 3-isobutyl-l-methylxanthine (IBMX, from Sigma-Aldrich), an inhibitor of cAMP phosphodiesterase, for 10 min followed by ligand treatment (10μM, all from Enamine) for 10 min. cAMP assay was performed using a colorimetric cAMP ELISA assay kit (Cell Biolabs) according to the manufacturer's instructions.

RNA extraction, cDNA synthesis and quantitative PCR

Rat blood RNA was extracted using the Ribopure RNA Purification kit (Life Technologies). One μg of total RNA was reverse transcribed using the SuperScript III first-strand synthesis kit (Life Technologies). Quantitative PCR (qPCR) was performed in four replicates in the 7900HT Fast Real-time PCR system (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used in qPCR assay are summarized in Table I. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method as previously described (Zhao et al. 2013b).

Assessment of tau phosphorylation

Twenty μg protein lysate of either primary neuron or rat hippocampus samples were used for SDS-PAGE protein separation. Separated proteins were transferred onto nitrocellulose membrane, and western blotting was performed using 5% milk in Tris-buffered saline solution for blocking nonspecific binding sites and antibody dilutions. Primary antibodies against tau proteins included mouse monoclonal antibodies as follows (location of epitopes refers to the longest tau isoform of 441 amino acid residues): AT8 (pSer202/205), AT100 (pThr212/Ser214) and PHF-1 (pSer396/404).

For assessment of active JNK, anti - phospho-JNK (Cell Signaling) was used as probe and β-actin signaling (Santa Cruz Biotechnology) was used controlled for samples loading.

Statistics

In these studies, all values are expressed as mean and standard error of the mean (SEM). Differences between means were analyzed using one-way ANOVA or two-tailed student t-test. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using the Prism Stat program (GraphPad Software, Inc.).

Results

Modeling of OR4M1

A model of the 3-D structure of olfactory receptor OR4M1 was built by comparative modeling based on the crystal structure of bovine rhodopsin (Okada et al. 2004). Most of the predicted binding site residues are spatially close in the model suggesting that the alignment is reliable. The alignment shows that the region between Ala 142 and Pro 175 of OR4M1 is highly divergent from the equivalent region in rhodopsin, hence this region was further refined using via loop modeling. In the final model most of the predicted binding site residues from Man *et al*. (Man, Gilad, & Lancet 2004b) cluster into one region (Fig. I) which is consistent with a binding pocket identified in the model using program SiteHound (Ghersi & Sanchez 2009a).

Docking and in silico screening

The pocket identified in the OR4M1 model was used as a target for virtual screening of a set of ~ five million lead-like commercially-available compounds (Irwin & Shoichet 2005a). The top 700 hits from the screening (ranked by docking score) were visually inspected, and 57 compounds, which showed good occupancy of the predicted binding pocket and at least two hydrogen bonds with the protein, were selected, shown as a space filling model (Fig. IIA) and with predicted binding pocket (red mesh) (Fig. IIB).The selected compounds were clustered based on chemical similarity. This resulted in 32 clusters of structurally similar compounds. Based on the availability of these compounds, we chose 25 commercially available compounds that could be further grouped into 16 clusters of structurally similar compounds (Table II) for experimental validation.

In vitro validation of candidate OR4M1 ligands

In this study, embryonic cortico-hippocampal neuronal cultures from Tg NSE-OR4M1 mice were used as test candidates' compound to activate OR4M1. The intracellular signaling pathways activated by GPCR signaling include the cAMP/JNK pathway among others. Since OR4M1 belongs to the GPCR family, we treated TgOR4M1 neuronal cultures with individual ZINC ligands at 10 μM concentration and assessed for OR4M1-mediated cellular induction of cAMP as an index of OR4M1 activation.

We found that among the 25 compounds tested, ZINC10915775 was particularly efficient in the activation of OR4M1 as indicated by increases in cellular cAMP contents in response to ligand treatment (24 hr) (Table II). These ring structures occupied the two wider regions in the OR4M1 pocket, while the linker occupied the narrow connecting region (Fig. III A, B).

Based on this evidence, we continued to test the dose-responsiveness of activation of OR4M1 by ZINC10915775 in E15 cortico-hippocampal cultures generated from OR4M1 transgenic mice. Using the cAMP assay, we found that the activation of OR4M1 by ZINC10915775 reached peak activity at 100 nM (Fig. IIIC), so we chose this concentration for further *in vitro* mechanistic studies.

OR4M1 ligand ZINC10915775 inhibited tau phosphorylation through inhibition of c-Jun N-terminal kinase (JNK) signaling

Based on this evidence we continued to explore whether OR4M1 activation could influence abnormal tau processing. In this study cortico-hippocampal neuron cultures derived from either TgOR4M1 mice or WT littermates ZINC10915775 (100 nM for 12 hrs). We found that treatment of TgOR4M1 neurons with the ZINC10915775 compound significantly decreased tau phosphorylation on Ser202/T205 (AT8 epitope) and Thr212/Ser214 (AT100 epitope), but not on Ser396/404 (PHF-1 epitope) (Fig. IVA).

Interestingly, we also found that attenuation of abnormal tau phosphorylation in the TgOR4M1 cortico-hippocampal neuron cultures in response to ZINC10915775 treatment coincided with a significant decrease in the levels of phosphorylated JNK (Fig. IVA).

However, no detectable change in tau phosphorylation or JNK was detected in WT neuron cultures upon ZINC10915775 treatment (Fig. IVB), suggesting that ZINC10915775-mediated activation of OR4M1could lead to decreased tau phosphorylation, possibly through the inhibition of the JNK signaling pathway.

Expression of OR and tau phosphorylation in a rat model of blast-induced TBI (bTBI)

To examine whether the down-regulation of OR4M1 as well as the expression of three other ubiquitously expressed OR previously found decreased in subjects with histories of TBI (Zhao et al. 2013c) we explored the expression of the rodent OR orthologs in an *in vivo* rat model of bTBI for potential future *in silico* drug screening studies.

Excitingly, we found that similar to what was found in subjects with a history of TBI, the expression of Olr1612, Olr1671, Olr322 and Olr735 which are the rodent orthologs of the human, OR4M1 OR2J3, OR4D9 and OR4X1, respectively, showed a time–dependent decrease in PBMCs at 1, 3, 6, and 12 months in the same rat group post-bTBI compared to naïve control rats (Fig. V).

Discussion

The functional limitations and costs related to traumatic brain injury (TBI) place a profound burden on individuals and their families in the United States military and civilian communities (Leibson et al. 2012). With improved medical care, TBI morbidity and hospitalizations have declined 20% and 50% since 1980, but have resulted in more long-term morbidity and disability (Thompson, McCormick, & Kagan 2006). Long term disability after TBI has been attributed to cognitive and neurological disorders. Given the association with cognitive and neurobehavioral changes, there is increasing speculation that TBI is associated with an increased risk of Alzheimer's disease and other tauopathies, including Lewy Body dementia (LBD), Frontotemporal dementia (FTD), among others. However, epidemiological studies linking TBI and ADRC have resulted in conflicting data. Some studies have reported an association between TBI and an increased risk for developing Alzheimer's disease and related conditions, including tau misfolding and abnormal processing on of β-amyloid (Barnes et al. 2014;Bazarian et al. 2009;Graves et al. 1990;Guo et al. 2000;Molgaard et al. 1990;Mortimer et al. 1991;Wang et al. 2012), but many others have found no such risk (Chandra et al. 1989;Himanen et al. 2006;Mehta et al. 1999;Millar et al. 2003;Williams et al. 1991).

The current study is based on our recent observation that abnormal tau processing in subjects with a history of TBI may be traced back to abnormal down-regulation of certain ORs, including OR4M1 and OR11H1, in the brain (Zhao *et al.*, 2013). In this study, we demonstrated that activation of OR4M1 with select ligands led to reduction of phosphorylation at Ser202/205 and Thr212/Ser214 of tau and found that the reduction of tau phosphorylation was associated with altered activation of the JNK pathway. Interestingly the physiological relevance of OR4M1 is consistent with the recent study demonstrating that controlled cortical impact TBI activates JNK and increases tau phosphorylation in a 3xTg mouse model of AD (Tran *et. al.,* 2013).

To explore the cause-effect relationship between TBI and OR down-regulation, and TBI-type neuropathology, we used a bTBI rat model. Consistent with our observation in human TBI subjects (Zhao *et. al.,* 2013), we found significantly lower contents of OR4M1, and other select ORs previously found in the blood of blast-injured rats compared to control rats over 12 months following TBI exposure.

Our studies have identified a novel ligand for OR4M1 TBI biomarker through *in silico* screening, and activation of OR4M1 by this ligand in primary neuronal cultures resulted in decreased tau phosphorylation through inhibition of the JNK signaling pathway. Taking these findings together, our hypothesis is that inactivation of certain ubiquitous ORs in response to conditions associated with TBI might be at the basis for potential abnormal tau phosphorylation possibly through mechanisms associated with the JNK pathway (Fig. VI). This evidence suggests a potential fine-tuning character of OR-mediated JNK regulation in response to conditions associated with TBI.

The sequence of early tau phosphorylation suggests that there are events prior to filament formation that are specific to particular phosphorylated tau epitopes, leading to conformational changes and cytopathological alterations. There is evidence that a healthy neuron develops immunoreactive punctate phospho-tau inclusions, primarily AT8 and AT100, which eventually become preNFTs which give rise to a filamentous intra-cellular inclusion. Eventually the neuron dies and an extra-neuronal NFT, or ghost tangle remains. Our evidence that the ZINC10915775 may attenuate AT8 and AT100, which are indexes of the early stages of NFT formation, through the activation of OR4M1, supports the hypothesis that certain ligands of ubiquitous OR expression could be developed to target the very early stages of TBI associated ADRC, such as tauopathy (Augustinack *et al.* 2002) .

Our observation from the experimental bTBI model demonstrated, for the first time, a direct cause-and-effect relationship between long-term down regulation of select ORs and a neuropathological feature in TBI. This validates our hypothesis that down regulation of select ORs in PBMCs and in the brains of our human TBI study cohort may be caused by prior TBI exposure (Zhao *et al*., 2013). Our 3-D *in silico* screening model provides an innovative means to identify novel compounds that can modulate tau phosphorylation through activation of ORs. Future studies will focus on structure-activity relationships (SAR) around the active compound and the optimization of an *in silico* screening system based on this

information to identify compounds with higher potency and specificity as potential preventative and possibly therapeutic agents for TBI associated ADRC at early stages.

Conflict Of Interest

The authors declare no conflict of interest.

Acknowledgements

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Legend

Table I. List of primers used in qPCR assays.

Figure I. *In silico* 3-D model of OR4M1 and predicted binding pocket.
The predicted ligand binding residues from Man *et al*. (Man, Gilad, & Lancet 2004c) are shown in yellow. The predicted binding pocket identified by SiteHound (Ghersi & Sanchez 2009c) is shown as a red mesh. Additional residues that contribute to the binding pocket, but are not identified in Man *et al*. are shown in green.

Figure II. Example of virtual screening hit.
In panel A, one of the top scoring compounds is shown in cyan as a space-filling model. Protein binding site side residues are colored as in Figure 1. In panel B, compatibility of the same top scoring compound is shown with predicted binding pocket (red mesh).

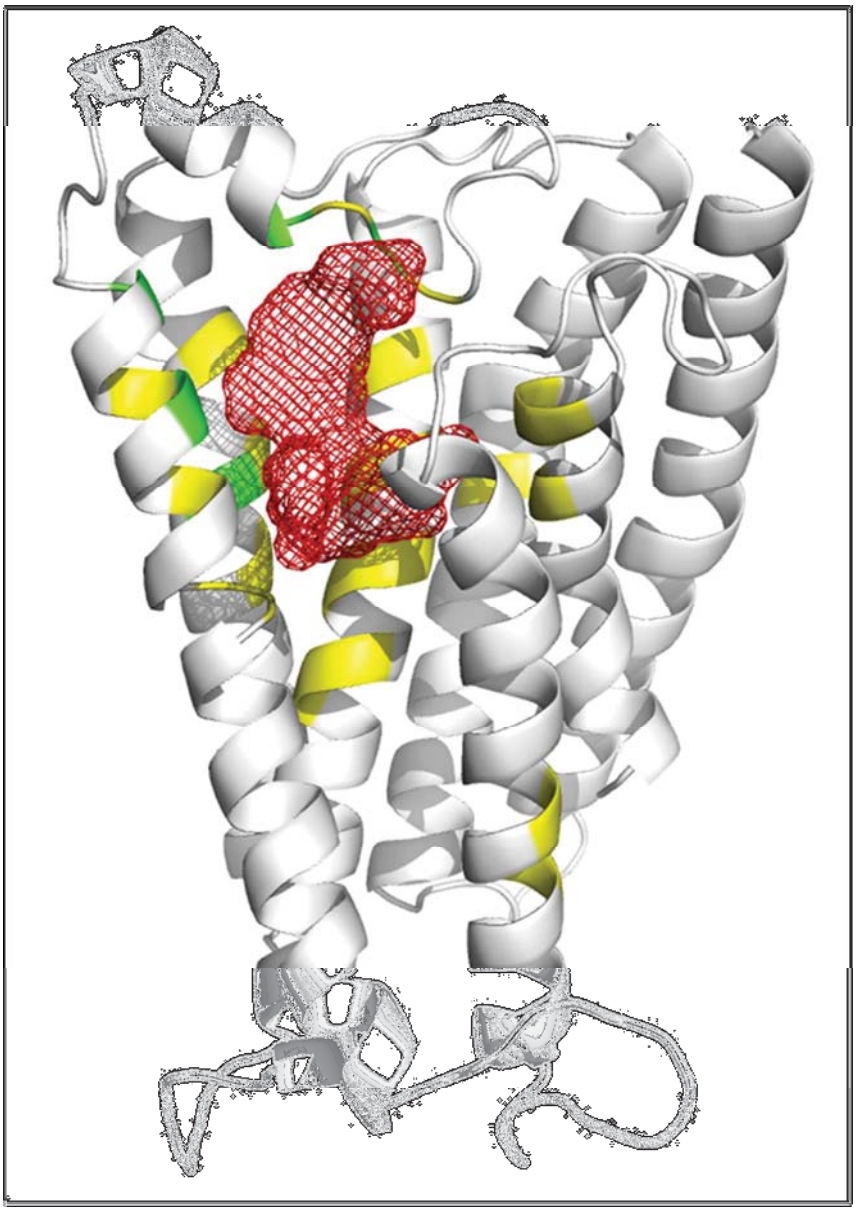
Table II. Experimental validation of OR4M1 ligands identified by *in silico* screening; two-tailed t-test
*P< 0.05 vs vehicle control

Figure III. Structure, docking of the two experimentally validated hits in the OR4M1 binding pocket and dose response assay.
In panel A, structure of ZINC10915775; in panel B, predicted binding position for ZINC10915775 compound in the OR4M1 binding pocket; in panel C, dose dependent activation of OR4M1 by ZINC10915775. In panel C, the statistical analysis for the sigmoidal curve was generated by Prism Stat program (GraphPad Software, Inc.); n=3-4 per culture condition.

Figure IV. OR4M1 ligand ZINC10915775 decreased tau phosphorylation and inhibited JNK signaling in primary neurons.
Embryonic day 15 cortico-hippocampal neuronal cultures from TgOR4M1 mice (A) and wild type mice (B) were treated with 100 nM ZINC10915775 for 1 hour and western blot analysis were performed to assess tau phosphorylation and JNK signaling. *p<0.05, **p<0.01, ***p<0.001, by two-tailed t-test; n=3-4 per culture condition.

Figure V. Long term effect of blast on the peripheral olfactory receptor levels in a blast-induced rodent model of TBI.
Blood was withdrawn at 1, 3, 6, and 12 months following the blast. Quantitative PCR was performed using ABI 7900HT Fast Real-Time PCR System using primers specific for (A) Olr1612; (B) Olr1671; (C) Olr322; (D) Olr735. *p<0.05, n=4-5 rats per group by 2-tailed t-test.

Figure VI. Scheme of working hypothesis.



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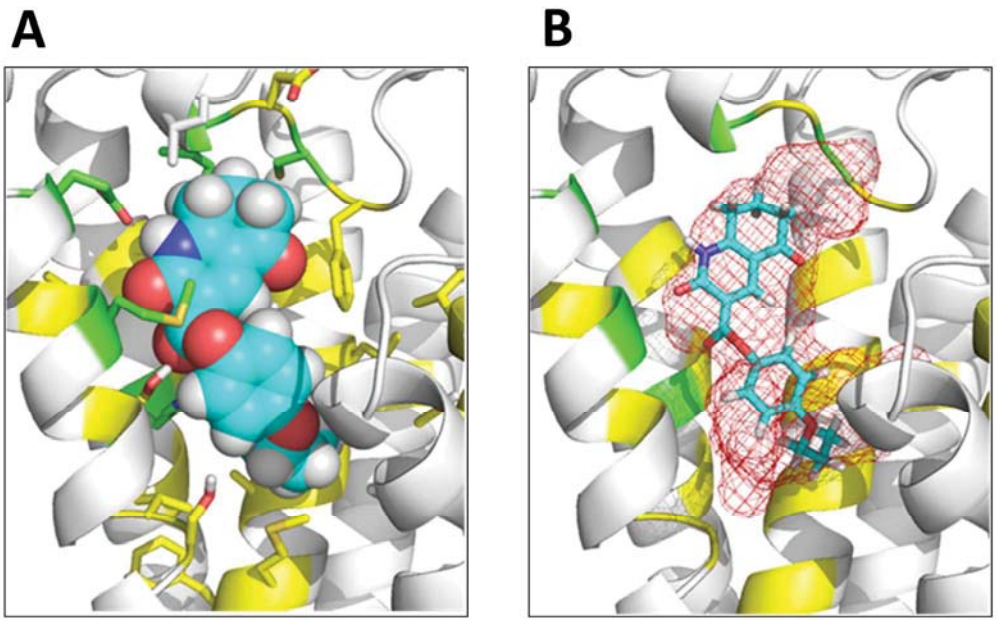

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In panel A, one of the top scoring compounds is shown in cyan as a space-filling model. Protein binding site side residues are colored as in Figure 1. In panel B, compatibility of the same top scoring compound is shown with predicted binding pocket (red mesh).
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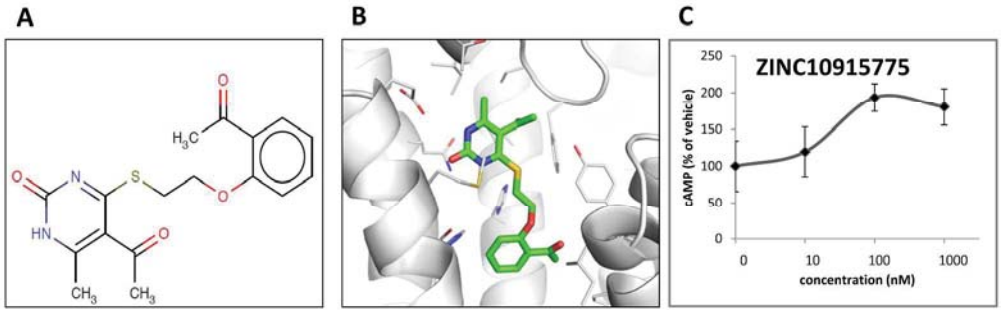

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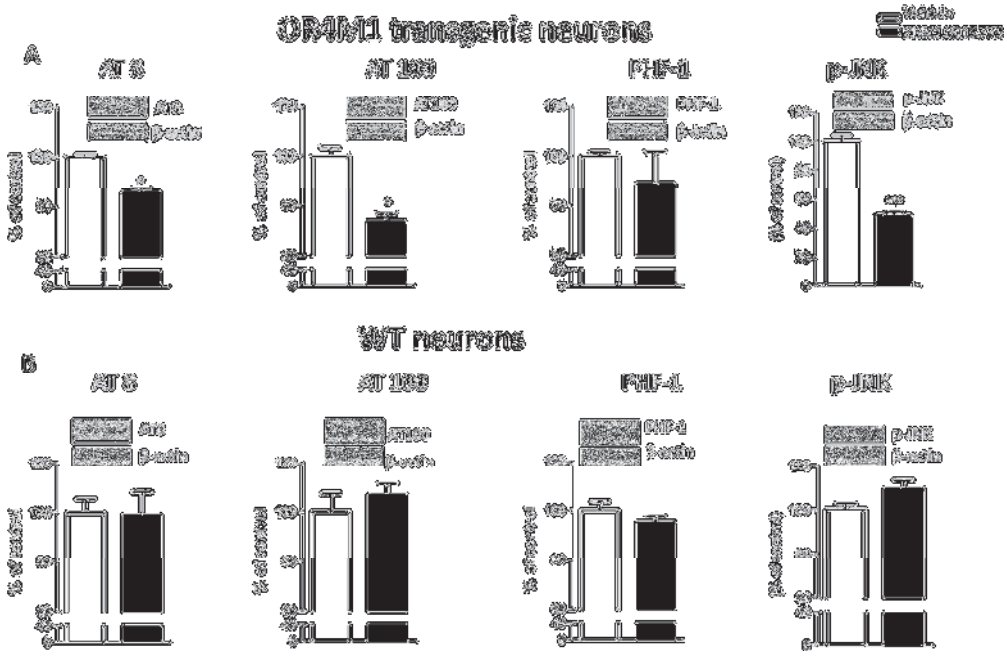

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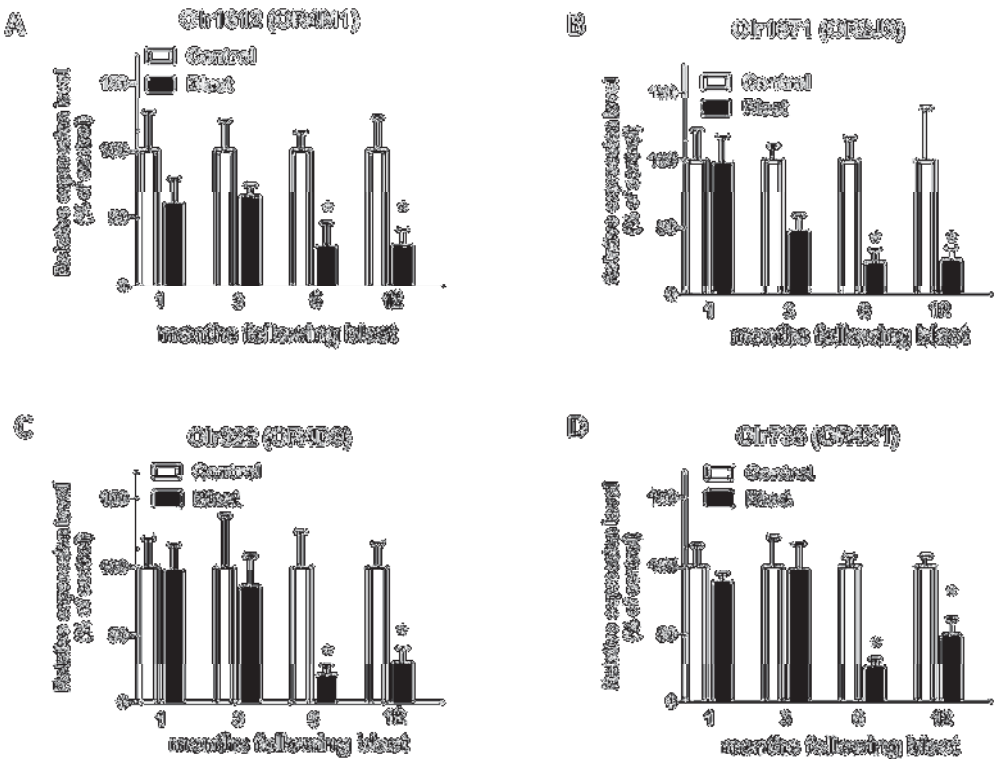

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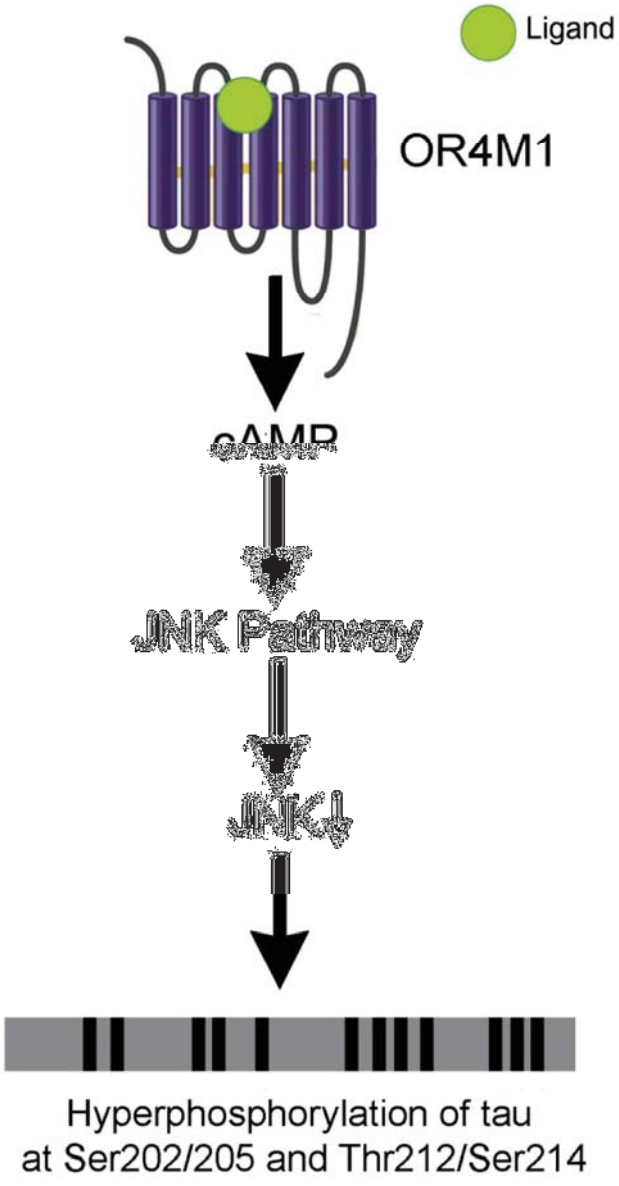

Figure VI. Scheme of working hypothesis.
72x136mm (300 x 300 DPI)

Gene	Forward	Reverse
Olr322	AAACTACACCAGGGTCAAAGAA	CACCAGGAGAAGGAAGAGAAAC
Olr735	GACCCATCCAGCACATTGAT	GGTTGGTGAGAGGAGAATTGAG
Olr1612	GCCTGTGCCAATACTTTTCTG	CTTGAGTGTTTCTTGAGCATGG
Olr1671	GTGATGTCCTATGACCGCTATG	TGAAGTGCTGAGGTGGTAAAG
TBP	CACCAATGACTCCTATGACCC	CAAGTTTACAGCCAAGATTCACG

Cluster	ZINC ID	cAMP (% of control) Mean ± STD
C1	00387281	72.37 ± 11.49
C2	12916861	81.38 ± 23.31
	32740605	113.81 ± 14.83
	09255703	115.59 ± 25.83
	9578142	88.90 ± 22.88
	13021362	89.33 ± 36.94
	09912561	63.98 ± 19.14
C3	69351464	65.20 ± 23.20
C4	12974343	71.82 ± 33.32
	05352464	92.27 ± 23.98
C5	14148934	107.37 ± 25.22
	14149349	119.59 ± 39.55
	10913993	134.95 ± 28.33
	10913792	102.15 ± 14.23
C6	03437366	70.56 ± 13.00
C7	12768603	62.32 ± 20.52
C8	71896059	47.75 ± 10.34
C9	05937266	90.38 ± 3.98
C10	71821329	49.72 ± 3.08
C11	05455334	62.70 ± 6.34
C12	65538929	40.86 ± 5.88
C13	08987000	61.39 ± 10.79
C14	30481138	82.90 ± 15.93
C15	10915775	261.89 ± 39.03*
C16	32777649	428.61 ± 46.10*